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PRESENT CONDITION OF A STRAIN OF CONNECTIVE TISSUE TWENTY-EIGHT MONTHS OLD.*

By ALEXIS CARREL, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

PLATES 1 AND 2.

In previous articles[1] it was shown that connective tissue could be kept outside of the organism in a condition of permanent life. The purpose of the following experiments was to determine the present condition of a strain of connective tissue which, after having undergone 358 passages, has now reached the twenty-ninth month of its life *in vitro*. The strain of connective tissue was derived from a piece of heart extirpated on January 17, 1912, from a chick embryo seven days old. The fragment of heart pulsated for 104 days and gave rise to a very large number of connective tissue cells. These cells multiplied actively during the last two years, and produced a large amount of connective tissue. At present, a great many cultures are obtained from the strain every week.

The dynamic condition of a tissue is manifested by the rate of its growth. The increase in the volume of a fragment of connective tissue can be measured with comparative accuracy. For this the following technique is used. A fragment of tissue is removed from a culture, washed in Ringer solution, and placed in a new medium. It soon becomes surrounded with a ring of new tissue. After forty-eight hours the width of this ring is measured with a micrometer. Under the ordinary conditions of the experiment the thickness of the new tissue is more or less uniform, and its total volume can therefore be estimated fairly accurately by its superficial size. The fragments of tissue usually double in forty-eight hours. But their rapidity of growth is subject to fluctuations dependent upon the character of the medium and upon the condition of the tissue at the time that it is placed in the medium. When these conditions were favorable the ring of new tissue attained, during

* Received for publication, May 10, 1914.

[1] Carrel, A., *Jour. Exper. Med.*, 1912, xv, 516. Ebeling, A., *idem*, 1913, xvii, 273. Carrel, A., *idem*, 1913, xviii, 287.

the last few months, a width of 2 to 2.8 millimeters (figures 1 and 2). A comparison of the amount of tissue produced by a given culture in forty-eight hours this year with that produced in the same time by the same strain of cells a year ago shows that the activity of the strain has increased. Last year the width of the ring of tissue produced in forty-eight hours around the fragments of connective tissue was only 1.5 or 1.8 millimeters.

This increase in the rate of growth is made more apparent by the following experiment. A piece of heart extirpated from a chick embryo eight days old, and a fragment of connective tissue at the beginning of the third year of its life *in vitro* were placed in the same culture medium. After forty-eight hours it was seen that the tissue which had become adapted to the life *in vitro* had increased much more rapidly than the fresh tissue (figure 3). Nevertheless, the tissue adapted to the life *in vitro* was derived indirectly from a fragment of heart extirpated more than two years ago from an embryo seven days old. Thus it is conclusively shown that the proliferating power of the strain has in no wise diminished. Nevertheless, it would be imprudent to conclude from this fact that it has augmented, as the greater rate of increase of the tissues may be due, not to an augmentation of the proliferating power of the cells, but to an improvement in the details of the technique.

Moreover, the fact remains that during the third year of independent life the connective tissue shows greater activity than at the beginning of that period, and is no longer subject to the influence of time. If we exclude accidents, connective tissue cells, like colonies of infusoria, may proliferate indefinitely.

EXPLANATION OF PLATES.

Plate 1.

Fig. 1. A fragment of connective tissue extirpated from a culture of the twenty-eight months old strain, one hour after the passage.

Fig. 2. The same tissue, forty-eight hours after the passage.

Plate 2.

Fig. 3. In the same medium were placed a piece of heart from a chick embryo eight days old (A), and a fragment of connective tissue, No. 8860, which had lived for more than two years outside of the organism (B). The photograph shows the amount of tissue produced in forty-eight hours by both fragments.

PATCHING AND SECTION OF THE PULMONARY ORIFICE OF THE HEART.*

By THEODORE TUFFIER, M.D., and ALEXIS CARREL, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

The purpose of these experiments was to develop a technique by means of which the pulmonary orifice could be enlarged. The operation consisted in suturing to the anterior side of the orifice a venous patch which permitted an increase in the circumference of the orifice after the arterial wall had been incised.

TECHNIQUE.

The animals were etherized according to the Meltzer-Auer method. The thoracic wall was shaved with sodium sulphide and sterilized with iodin, and the animal was put on an electric warming pad. The thoracic cavity was then opened on the left side by a large transverse incision which was kept widely open by means of a Gosset retractor. The operating field was walled off by means of a technique precluding the occurrence of pleurisy, which has already been described.[1] The pericardium was opened through a long incision made on the right side of the phrenic nerve at about two centimeters' distance from the nerve, and the anterior part of the heart and of the pulmonary artery was exposed.

The position of the valves was easily located. The wall of the pulmonary artery is thinner at the level of the sigmoid sinuses and its color is slightly darker. This point was selected for the lower end of an incision of about 1.5 centimeters made through the arterial wall above the anterior sigmoid valve. It was found afterwards that this incision was too short, and that when it was longer it produced a very marked pulmonary insufficiency. In order to be more efficient and less dangerous, the section of the orifice should be

* Received for publication, May 10, 1914.

[1] Carrel, A., *Surg., Gynec. and Obstet.*, 1914, xix, (in press).

made at the point of insertion of the anterior and left sigmoid valves on the arterial wall. After the location of the incision had been selected the patch was applied on the anterior wall of the pulmonary artery and of the heart. This patch was usually a piece of vena cava which had been preserved in cold storage for a few days. Pieces of artery could also be used, but as the arterial wall is thicker and harder than the venous wall it was more difficult to fix the patch to the pulmonary wall without danger of leakage. The shape of the patches was generally rectangular, about 2.5 by 2.5 centimeters. The patch was fixed to the pulmonary artery and to the heart by four stitches. The stitches of the lower and upper sides of the rectangle were about 2.5 centimeters distant from each other. The right and left sides of the patch were placed in such a way that the distance between the right and left lines of suture was only about 1.5 centimeters. As the width of the flap was 2.5 centimeters this allowed of one centimeter's increase in the circumference of the pulmonary orifice after it had been cut. The right upper and left sides of the flap were fixed to the anterior wall of the pulmonary artery by a continuous suture. Between the lower end of the flap and the wall of the heart there was a free space through which the incision of the orifice could be made. For this purpose special scissors were constructed, composed of one long sharp blade and one short blunt blade. The arterial wall was perforated with the long blade of the scissors just at the level of the pulmonary orifice. The long blade was pushed into the lumen of the pulmonary artery, and the wall was cut by introducing the short blade deep under the flap. There was an immediate escape of dark blood, the scissors were quickly removed and at the same time the index finger of the assistant compressed the flap against the opening made with the scissors, thus arresting the hemorrhage. Then the lower side of the flap was rapidly united to the cardiac wall by means of a continuous suture. This stage of the operation was generally made without interrupting the circulation through the heart. The operation was facilitated by interrupting it for one or two minutes; but as the clamping of the pedicle of the heart is always somewhat dangerous, this procedure was not often used. After the suture had been completed slight compression with a

sponge was applied on the flap and on the lines of suture. If there was still some leakage after a few minutes, complementary stitches were added. The pericardium was never closed before the line of suture was absolutely secure against the escape of blood. After suture of the pericardium, the thoracic cavity was closed by the ligation of two ribs and by three or four planes of suture. Afterwards the dog was dressed and cared for by the customary methods.

EXPERIMENTAL.

PATCHING OF THE PULMONARY ARTERY.

The experiments were performed on eight medium sized dogs. The technique for the first four operations was in its developmental stage and differed slightly from that used for the last four operations.

Experiment 1.—Black female dog, medium size. Oct. 23, 1913. Etherization by the Meltzer-Auer method. Transverse thoracotomy. Ligature of both mammary arteries and section of the sternum. Opening of both pleural cavities. Incision of the pericardium. On the anterior part of the pulmonary artery and of the cardiac wall was placed a fragment of aorta taken twenty-four hours previously from a fresh cadaver of a human fetus and preserved in cold storage. The upper and the lateral sides of the patch were fixed to the pulmonary wall by a continuous suture. Then the pedicle of the heart was clamped. A small knife was introduced under the patch and the wall of the pulmonary artery was cut. The incision was too short and too high. Next, the lower side of the patch was sutured to the cardiac wall, and the clamp was removed after the circulation had been interrupted for two minutes. Massage of the heart. After a few minutes the contractions were normal. Closure of the pericardium and of the thoracic cavity. No shock. After the operation the animal remained in normal condition. May 5, 1914. Animal in excellent health.

Experiment 2.—White dog, medium size. Oct. 26, 1913. Etherization by the Meltzer-Auer method. Opening of the thoracic cavity by the same method as that used in experiment 1. The patch was made of a fragment of dog's aorta preserved for twenty-four hours in cold storage. The clamping of the pedicle of the heart and the suture of the flap were performed as in the previous operation. The section of the wall of the pulmonary artery was made with fine scissors at the level of the insertion of the sigmoid valves. After the clamp was removed fibrillary contractions appeared and the animal died. An examination of the specimen showed that the wall of the pulmonary artery was cut as far as the insertion of the anterior valve. The opening was completely protected by the patch, but a small branch of the coronary artery had been taken up by a stitch. This was possibly the cause of the fibrillary contractions. The incision should have been made further to the left side of the artery, in order to cut the orifice at the point of insertion of the left and anterior valves.

Experiment 3.—Black and white bulldog, medium size. Oct. 31, 1913. Etherization by the Meltzer-Auer method. Transverse thoracotomy on the left side without section of the sternum and without ligation of the mammary arteries. A piece of dog's jugular vein preserved for twenty-four hours in cold storage was placed on the pulmonary artery. The lateral and the lower sides were fixed to the arterial and cardiac wall by a continuous suture. Then the wall was cut with scissors as far as the insertion of the sigmoid valves. Hemorrhage was prevented by compressing the flap to the opening with the index finger. Next, the upper side of the flap was sutured to the arterial wall. No massage of the heart. Normal pulsations. Closing of the pericardium and of the thoracic cavity. Two hours afterwards the animal was in excellent condition. May 5, 1914. Dog normal.

Experiment 4.—Female bulldog, medium size. Nov. 3, 1913. The same technique was used as in experiment 3. The patch was taken from a piece of vena cava preserved in cold storage since Oct. 27. It was sutured without the heart being clamped. No shock. The animal remained in good condition after the operation. Nov. 6. Animal ill. Nov. 8. Animal died.

Autopsy.—Purulent pericarditis. The space between the flap and the arterial wall was filled by a clot. The incision had healed. No fibrin or thrombus in the lumen of the vessel. As the incision was too short there was no increase in the circumference of the orifice.

Experiment 5.—Black and white male fox-terrier. Nov. 4, 1913. Etherization by the Meltzer-Auer method. Opening of the thoracic cavity and the pericardium with the same technique as that used in experiment 4. The patch was made from a piece of vena cava preserved in cold storage since Oct. 27. It was fixed on three sides and the anterior wall was opened with a cataract knife without any interruption of the circulation of the heart. Then the fourth side was sutured and the operation completed by the ordinary method. After the operation the dog remained in excellent condition. On Nov. 19 the animal had completely recovered. Strong diastolic murmur could be heard. May 1, 1914. Animal in excellent condition. The murmur had almost completely disappeared.

Experiment 6.—White female bulldog. Nov. 11, 1913. The technique used was identical with that of experiment 5. On Nov. 19 no murmur was audible. The animal remained in excellent condition, and is completely normal on May 5, 1914.

Experiment 7.—White male bulldog. Nov. 26, 1913. Etherization by the Meltzer-Auer method. The technique was identical with that used in the last two experiments. The section of the wall was made with special scissors composed of a long sharp blade and a short blunt one. The animal remained in excellent condition after the operation and is still normal on May 5, 1914.

Experiment 8.—White female bulldog. Dec. 2, 1913. Etherization by the Meltzer-Auer method. Technique identical with that used in the last three operations. The patch was made from a piece of jugular vein preserved in cold storage since Nov. 28. The animal remained in excellent condition and is still normal on May 5, 1914.

RESULTS.

The results of these operations must be considered from two standpoints: the danger to the life of the animal, and the modifications of the pulmonary orifice.

When the technique previously described was carefully followed, the operation was of little danger to the life of the animal. Although these operations were in the developmental stage, nevertheless only two animals died of the eight that were experimented upon. One of these died on the operating table from fibrillary contractions, probably due to the fact that a branch of the coronary artery had been taken up by one of the stitches. A second dog died of pericarditis a few days after the operation. It is probable that neither of these complications will occur again, since a better way of handling the heart has been found. In the six other instances the animals sustained no shock after the operation, remained in good health, and were still normal more than six months after the operation. The operation will be much less dangerous in the future, because the details of the technique have now been completely established.

The changes produced in the anatomy of the pulmonary orifice were examined on two specimens. In experiment 2 the dog died of fibrillary contractions on the operating table. The specimen showed that the incision had been too short and was located on the right side of the artery. The operation as it was performed was insufficient as well as dangerous. It was insufficient because the circumference of the orifice was not increased; and it was dangerous because the flap was fixed to the cardiac wall at a site where there were many branches of the coronary arteries. In experiment 3 the animal died of pericarditis. The flap had taken, but the incision was too short. The examination of the flap and of the incision demonstrated that no thrombosis had taken place, and that the patch of vein in contact on one side with dark blood and on the other with the pericardium, although in an unfavorable condition of nutrition, did not undergo necrosis. In the clinical examination of the six other animals it was found that only one presented a diastolic murmur a few weeks after the operation; that is, in one instance only was pulmonary insufficiency obtained. The lack of insufficiency in the other cases was due to the fact either that the incision of the pul-

monary artery was located too high and was too short, or that the flap was fixed too tightly to the arterial wall and did not allow of the dilatation of the orifice after it was incised. These details had to be learned in the course of the experiments, and it will be an easy matter to make an incision and patching which will permit a real dilatation of the orifice to take place. The incision must be about two centimeters in length, half being on the pulmonary artery and half on the ventricle, and it must cross the orifice at the point of junction of the left and the anterior sigmoid valves. The incision of the orifice and the suture of the fourth side of the flap will be made after the pedicle of the heart is clamped.

The six surviving animals are still in good health more than six months after the operation. In experiment 5 the diastolic murmur almost completely disappeared.

CONCLUSION.

These experiments show that it is possible to perform an operation, the object of which is to increase the circumference of the pulmonary orifice without involving much danger to the life of the animal. It is probable that operations of this type may come to be employed in the treatment of stenosis of the pulmonary artery in man.

EXPERIMENTAL OPERATIONS ON THE SIGMOID VALVES OF THE PULMONARY ARTERY.*

By ALEXIS CARREL, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

PLATES 3 TO 5.

The purpose of the following study was to ascertain whether and to what extent intracardiac operations could be performed with safety. The technique that has been described and employed by various experimenters and surgeons is unsatisfactory, because it involves the introduction of sharp instruments into the cavities of the heart, without the control of the eye or the finger. Thus far no operations have been devised which permit, for example, the cauterization of infected valves, the suture of the foramen ovale, or of two valves in a case of insufficiency, and still other plastic operations. The operations mentioned form a different class from those which have so far been performed, because they involve the stoppage of the circulation through the cavities of the heart and the passage of air into the cardiac cavities, and require great speed of execution. Hence it is hardly to be expected that a technique making possible and safe such operations as these will be perfected soon. It may even be regarded as extremely doubtful whether this class of operations may ever be applicable to human surgery. And yet their future cannot be predicted, and I have, therefore, attempted to develop methods for the execution of these operations. For the preliminary studies the sigmoid valves of the pulmonary artery were selected, because the opening of the right cavities of the heart is less dangerous than the opening of the left cavities.

METHODS.

The preparation of the animals is similar to that already described.[1] After the thoracic cavity has been opened in the anesthe-

* Received for publication, May 20, 1914.

[1] Tuffier, T., and Carrel, A., *Jour. Exper. Med.*, 1914, xx, 3.

tized animal by a left transverse thoracotomy, the pericardium incised, and the anterior part of the heart exposed, the operation is started. The operation consists of four stages: (1) the stoppage of the circulation of the heart, (2) the opening of the pulmonary artery, (3) the performance of the intracardiac operation and the closing of the pulmonary artery, and finally (4) the reëstablishment of the circulation through the heart.

The Stoppage of the Circulation of the Heart.—This has already been performed in many different ways. We ourselves have used all known methods of stopping the circulation through the heart. Finally, we adopted the method of clamping in mass the pedicle of the heart by means of large soft-jawed forceps. The heart was not taken out of the pericardium, but the incision of the pericardium was large enough to permit of the easy introduction of one of the jaws of the forceps under the pedicle. The forceps was a Doyen, the jaws of which were covered with rubber. One of the jaws was introduced into the pericardium under the pedicle and directed from the right to the left side by the index finger of the operator. Before clamping, the position of the forceps was carefully examined. The handling of the forceps and of the pedicle was always very gentle. Care was taken not to produce any compression of the veins before the time of the clamping. It is important for the heart to be in normal condition before clamping the pedicle, a result obtained by overventilating the blood, which is easily accomplished by means of the Meltzer-Auer apparatus. It was noticed that if the heart is clamped before it is in excellent condition the interruption of the circulation is less safe than when the heart is filled with well oxygenated blood. The advantage of using the Meltzer-Auer method in this operation is that it permits of an acceleration of the oxygenation of the blood at will. When everything is ready for the operation the forceps is rapidly clamped and, without a second being wasted, the heart or the vessel is opened and the operation started.

When these precautions are taken it is possible to clamp the pedicle of the heart for as long as two and a half or three minutes without subsequent trouble. As soon as the clamp is removed the heart resumes pulsation, and after a short time the pulsations are again

normal. In no case was there any need of massaging the heart when the interruption of the circulation did not exceed two and one half minutes; this period probably allows of a considerable margin of safety and it would doubtless be possible to prolong the operation slightly without excessive danger. But two and one half minutes appear to be sufficient time for the performance on the valves of several operations.

The above technique may appear to be somewhat crude, but experience has shown that it is simpler than the separate clamping of the arterial and venous pedicles, and requires less handling of the heart, which is an important consideration. Moreover, traumatism of the anatomical structures of the pedicle is slight, on account of the large quantity of tissue which is taken up between the jaws of the forceps and which renders their action less rough.

The Opening of the Pulmonary Artery.—The pulmonary orifice is exposed by means of an incision made through the anterior wall of the artery at the level of or a little above its junction with the heart. The incision is made with sharp scissors, or is begun with sharp scissors and finished with blunt scissors. The wall of the pulmonary artery is perforated about one and one half or two centimeters above the point of insertion of the sigmoid valves and incised from above downwards with one cut of the scissors. The location and the length of the incision vary according to the purpose. When the incision is located above the anterior valve it is not prolonged below the level of the insertion of the valve, in order not to incise the sigmoid. When the incision is made further on the left side of the artery at the level of the junction of the anterior and left sigmoids of the valve it can be prolonged further on to the cardiac wall, and an incision four centimeters in length, half on the heart and half on the pulmonary artery, could be made without danger. The short incision made above the orifice is kept open by two forceps put on the edges, when the valves are sufficiently widely exposed to be cauterized. But through such an opening it is impossible to perform a suture of the valves. Hence for these cases a long incision is made at the union of the anterior and left sigmoids (figures 1 and 2), which permits a large opening of the pulmonary artery and the possibility of operating easily on the valves

themselves. It is not necessary to cut exactly at the point of insertion of the valves. If the incision is located near the point of insertion of the valves it does not produce a marked degree of insufficiency. Moreover, the left side of the arterial cone is not covered by large branches of the right coronary artery, and the section of the wall and its suture can be made without danger to the circulation.

Immediately on opening the pulmonary artery a large quantity of dark blood is expelled from the heart, and consequently the operating field must be narrowly walled off by the silk and cotton padded towels, in order to prevent the escape of the blood into the pleura.

The Performance of the Intracardiac Operation and the Closing of the Pulmonary Artery.—As soon as the incision is complete its edges are retracted by two mosquito forceps, the blood is sponged, and the valves are exposed. Air always enters the right ventricle. No special care is taken of this, since no complications due to the air emboli through the lungs were ever observed to follow. Three kinds of operation were performed: cauterization, suture and section, and suture of the valves. The cauterization of the valves was performed with the fine point of the thermocautery. The points of insertion of the valves, their free edge, or their internal surface were cauterized. The suture of the valves was made with a straight needle No. 16 and fine silk sterilized in vaselin. The left and right valves were united by one stitch (figure 3) at a distance of about two millimeters from the insertion to the arterial wall. This produced stenosis of the pulmonary orifice. Section and suture of one sigmoid valve were made in the following way: The sigmoid valves having been exposed by a long incision through the orifice, the right sigmoid was cut in its middle with the scissors, as far as the insertion to the arterial wall (figures 4 and 5). Afterwards the edges of the wound were united near the margin of the valve by a stitch made with straight needles No. 16 and silk sterilized in vaselin.

The operation completed, the pulmonary artery is sutured. In order not to lose any time a needle No. 12 with China silk No. 1 was kept ready. A minute piece of muscle had been fixed at the end of the thread, in order that no time should be spent in making a knot after the first stitch was made; the incision is closed with a con-

tinuous through and through suture. The time occupied by the incision of the pulmonary artery, the operation itself, and the suture of the artery varied between one minute and fifty seconds and three minutes.

Reëstablishment of the Circulation through the Heart.—As soon as the suture of the pulmonary artery is complete the clamp is removed. Generally during the period of the interruption of the circulation the heart is still beating feebly, but it may have stopped completely. As soon as the blood is allowed to flow from the vessel into the heart the pulsations recommence, weak at first, but very soon become quite normal. When the interruption of the circulation did not last more than two or two and one half minutes there was no need of massage; after a few minutes the heart had recovered its normal pulsations. A gauze sponge was always applied to the line of suture and a slight degree of compression made during a few minutes. When, after that time, there was still some leakage at the line of suture, one or two complementary stitches were added. Care was taken not to close the pericardium before the line of suture was absolutely without leakage. Then suture of the pericardium and of the thoracic wall is made, according to the ordinary method. The animal is dressed and taken care of as described in a previous article.[2]

EXPERIMENTAL.

The experiments were performed on ten medium sized dogs. Nine of these animals were young adult dogs in good health, and one was a dog about seven or eight years old. In three instances the operations consisted of exploration, suture, or section and suture of the sigmoid valves; in seven instances the sigmoid valves were cauterized.

Experiment 1. Exploration of the Sigmoid Valves of the Pulmonary Artery.—Black long haired dog. March 10, 1913. Etherization by the Meltzer-Auer method. Transverse left thoracotomy by the ordinary technique. Incision of the pericardium. Clamping of the pedicle of the heart with a soft-jawed forceps protected with rubber. Incision of the anterior wall of the pulmonary artery just above the sigmoid valves. The edges of the opening were retracted with two forceps. The sigmoid valves of the pulmonary artery could easily be seen and

[2] Tuffier and Carrel, *loc. cit.*

handled with the fingers or with the forceps. Then the incision of the pulmonary artery was closed by means of a continuous suture with China silk and a needle No. 12. The clamp was removed and the circulation reëstablished. The interruption lasted two minutes and fifty seconds. The pulsation of the heart started immediately without massage. After a few minutes it was normal. Closing of the pericardium and of the thoracic cavity by the ordinary method. The animal had no shock and walked about half an hour after the operation. During the afternoon it ate and drank as usual. May 20. Animal is still entirely normal.

Experiment 2. Suture of the Sigmoid Valves of the Pulmonary Artery.—Black and white long haired male setter dog. March 12, 1914. Etherization by the Meltzer-Auer method. Transverse left thoracotomy by the ordinary method and clamping of the pedicle of the heart. Section of the wall of the pulmonary artery and of the cardiac wall at the point of union of the anterior and left sigmoid valves. The valves were widely exposed by retraction of the edges of the wound by two forceps, and the right and left sigmoids were united at a distance of about 2 mm. from their insertion to the wall by a stitch made with a straight needle (Kirby No. 16). Then the edges of the incision of the pulmonary artery were sutured by the ordinary method. The clamp was removed after an interruption of the circulation lasting two minutes and twenty-five seconds. The pulsation of the heart started immediately without massage. Then the pericardium and the thoracic cavity were closed. After the operation the animal recovered, as in experiment 1. May 20, 1914. Animal is still in normal condition.

Experiment 3. Section and Suture of the Right Sigmoid Valve and of the Pulmonary Artery.—Yellow and white male fox-terrier, about seven or eight years old. March 17, 1914. Etherization by the Meltzer-Auer method. Opening of the chest by left transverse thoracotomy. Section of the pulmonary artery just above the pulmonary orifice, after the pedicle of the heart had been clamped. The sigmoid valves were exposed by retraction of the edges of the incision by two forceps. Then the scissors were introduced into the artery and the right sigmoid valve was completely sectioned in its middle. Next, the edges of the valve were approximated at the upper end of the incision by one stitch made with a needle No. 16 and fine silk sterilized in vaselin. The pulmonary artery was closed by the ordinary method and the circulation was reëstablished after an interruption of two minutes and thirty seconds. The operation was completed in the ordinary way and the animal not only sustained no shock but remained in excellent condition. May 20, 1914. Animal still is in excellent health. Slight diastolic murmur.

Experiment 4. Cauterization of the Sigmoid Valves of the Pulmonary Artery.—Young brindle female bulldog. April 14, 1914. Etherization by the Meltzer-Auer method. Transverse thoracotomy and clamping of the pedicle of the heart by the ordinary method. Incision of the pulmonary artery just above the pulmonary orifice. The edges of the incision were retracted by two forceps and the sigmoid valves widely exposed. The blood was removed with sponges and the edges of the left and right sigmoid valves were cauterized with the thermocautery. Then the wall of the pulmonary artery was closed and the circulation reëstablished after an interruption of two minutes. The operation was completed by the ordinary method. The animal sustained no shock and remained

in excellent condition, as in the preceding experiments. May 20, 1914. Animal is still in normal condition.

Experiment 5. Cauterization of the Sigmoid Valves of the Pulmonary Artery.—Brindle female mongrel. April 20, 1914. Etherization by the Meltzer-Auer method. All the details of the operation were identical with those given in experiment 4. The interruption of the circulation lasted two minutes and five seconds. After the operation the animal remained in excellent condition. May 6, 1914. Animal is normal. May 8. Animal coughs. May 12. Died.

Autopsy.—Pneumonia, pericardial adhesions. Valves normal. Pulmonary incision healed, without deposit of fibrin.

Experiment 6. Cauterization of the Sigmoid Valves of the Pulmonary Artery.—Brindle female mongrel. April 22, 1914. Etherization by the Meltzer-Auer method. Transverse thoracotomy and clamping of the pedicle of the heart by the ordinary method. Incision of the pulmonary artery just above the middle part of the anterior sigmoid valve. The incision was made as far as the base of the sigmoid sinus. Cauterization of the anterior sigmoid and of the point of insertion of the right and left sigmoids. Suture of the pulmonary artery and reëstablishment of the circulation after an interruption of two minutes and fifty seconds. Pulsation of the heart weak at first, normal after a few minutes. During the suture of the pericardium a small branch of the anterior coronary artery was wounded by the point of the needle. The hemorrhage was stopped by suture of the endocardium in front of the vessel. Hemorrhage ceased. The operation was completed in the ordinary way. The animal had no shock and in the afternoon was in excellent condition. April 28. Animal sick. April 29. Died.

Autopsy.—Wound infection; purulent pleurisy; no pericarditis, but pericardiac adhesions. Interior of heart normal; no thrombosis of pulmonary artery; thin layer of fibrin on the lower part of the line of suture; sigmoid valves normal; no deposit of fibrin at cauterized points (figure 6).

Experiment 7. Cauterization of the Sigmoid Valves of the Pulmonary Artery.—White and black mongrel. April 23, 1914. Etherization by the Meltzer-Auer method. The technique of the operation was identical with that used in experiment 6. But after the circulation had been interrupted and the pulsation of the heart had recommenced, fibrillary contractions suddenly appeared and the animal died. The fibrillary contractions were probably due to the fact that the incision had been made too low on the anterior part of the heart and that a deep stitch possibly included a branch of the coronary artery.

Experiment 8. Cauterization of the Sigmoid Valves of the Pulmonary Artery.—White female fox-terrier. April 28, 1914. Etherization by the Meltzer-Auer method. Incision of the skin at 10.10 A.M. Opening of the chest by transverse thoracotomy on the left side at 10.18. The pedicle of the heart was clamped at 10.30. The incision of the pulmonary artery, the cauterization of the valves, and the closing of the pulmonary artery were performed with the ordinary technique. The interruption of the circulation lasted one minute and fifty seconds. The operation was completed at 10.45. The animal was in excellent condition. May 20. Animal is still normal.

Experiment 9. Cauterization of the Sigmoid Valves of the Pulmonary Artery.—White and yellow male fox-terrier. April 29, 1914. Etherization by the Meltzer-

Auer method. Transverse thoracotomy. The heart was clamped for two minutes, during which time the incision of the pulmonary artery, cauterization of the valves, and suture of the arterial wall were made. The operation was completed and the dressing made. Same technique as in experiment 6. Animal was in excellent condition. May 20. Animal still normal.

Experiment 10. Cauterization of the Sigmoid Valves of the Pulmonary Artery.—White fox-terrier with brindle spots. April 30, 1914. Etherization by the Meltzer-Auer method. The technique was exactly the same as in experiment 9. At 11 A.M. incision of the skin. At 11.05 opening of the chest. At 11.09 opening of the pericardium. At 11.15 clamping of the heart, which lasted one minute and fifty seconds. During this period the pulmonary artery was cut, the valve cauterized, and the arterial wall sutured. At 11.27 the suture of the muscles was made, and at 11.32 the operation was completed. The animal was in normal condition. May 20. Animal is still normal.

RESULTS.

The results of these operations must be considered from the standpoint both of the general condition of the animals and of the modifications of the sigmoid valves of the pulmonary artery. Out of ten animals operated upon, three died and seven recovered and remained in excellent condition. The deaths were due to different causes. In experiment 5 the animal recovered completely and was apparently normal sixteen days after the operation. Then it sickened and died of pneumonia twenty days after the operation. In experiment 6 the cause of death which occurred seven days after the operation was a purulent pleurisy. In experiment 7 the animal died on the operating table of fibrillary contractions of the heart. It is probable that some of the causes of death can be eliminated. The infection of the thoracic wall and the purulent pleurisy, as well as the pneumonia which occurred in experiment 5, are preventable complications. They were caused by faulty technique which can be avoided. In all instances in which a few persons only were present in the operating room, the operation could be conducted with great care and all the animals recovered. It is probable that the proportion of deaths obtained in the first series of experiments will be much lower in the future. The general condition of the animals that survived was excellent. They sustained no shock; usually one hour after the operation they walked about, and three or four hours afterwards they ate and drank. These results were observed not only on young dogs, but also on a dog seven or eight

years old. All the animals were operated upon more than one month ago, and are at present normal.

The local modifications brought about by the operations were studied on the two specimens taken from animals 5 and 6, and also from the clinical examination of the seven surviving animals. The heart taken from animal 6 showed the result of the cauterization of the sigmoid valves of the pulmonary artery seven days after the operation. There were pericardiac adhesions on the anterior wall of the heart, but the heart itself was apparently normal. No thrombosis of the pulmonary artery existed. Above the anterior valve was the cicatrice of an incision one and one half centimeters long, which extended almost to the bottom of the sigmoid sinus (figure 6). The lower part of the incision was covered by a thin film of fibrin. In the upper part the stitches could be seen; the union of the edges of the wound was perfect. The margin of the anterior valve was irregular, owing probably to the fact that it had been cauterized. But there was no apparent deposit of fibrin. The left and right valves were normal. In experiment 5 the specimen showed the result of the operation after twenty-two days. The anterior and posterior parts of the heart were almost completely adherent to the pericardium. The cicatrice of the incision was seen just above the anterior valve near its left insertion in the anterior wall. It was entirely cicatrized, the stitches could not be seen, being covered by a smooth, glistening membrane. The margin of the right sigmoid was slightly irregular, owing probably to the cauterization. The two other sigmoids were normal. The examination of the two specimens demonstrated that it was possible to make a section of the wall of the pulmonary artery without injuring the valves, and that the cauterization of the valves does not produce thrombosis.

The condition of the heart of the seven animals that remained in good health was examined clinically. Six of the animals were entirely normal. In experiment 3 the animal presented a slight diastolic murmur. This animal had undergone a section of the right sigmoid valve and suture of the valve by one stitch placed near the margin. It is probable that the bottom of the valve did not unite and that a slight amount of insufficiency persisted.

CONCLUSION.

Incision, suture, and cauterization of the sigmoid valves of the pulmonary artery have been performed successfully in dogs. In the first series of ten animals, there were only three accidents, probably from largely preventable causes, leading to the death of the animals.

EXPLANATION OF PLATES.

PLATE 3.

FIG. 1. Incision of the pulmonary artery at the union of the anterior and left sigmoid valves.

PLATE 4.

FIG. 2. Same as figure 1.

FIG. 3. Suture of the right and left sigmoid valves.

FIGS. 4 and 5. Section and suture of the right sigmoid valve.

PLATE 5.

FIG. 6. Specimen taken seven days after the cauterization of the sigmoid valves.

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THE RELATION OF THE SPLEEN TO BLOOD DESTRUCTION AND REGENERATION AND TO HEMOLYTIC JAUNDICE.

IX. The Changes in the Bone Marrow after Splenectomy.*

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In connection with the various investigations[1] carried out in this laboratory on the subject of the spleen in its relation to blood destruction and regeneration, the bone marrow of splenectomized dogs has been examined with a view to determining the compensatory or other changes following the removal of the spleen. As the material now consists of marrows representing periods varying from three weeks to twenty months after splenectomy, we consider our study sufficiently comprehensive to justify a detailed report.

In the literature of the subject, the references to changes in the bone marrow following splenectomy are for the most part casual and presented but incidentally in connection with the associated changes in the lymph and hemolymph glands. In Warthin's (1) collection of the literature up to 1903 the following references occur: Tizzoni and Fileti (2) (1880) and Tizzoni (3) (1882) observed in splenectomized dogs a transformation of the fatty marrow of long bones into red marrow. Mosler (4) (1882), working likewise with dogs, concluded that following splenectomy there may be compensatory action on the part of both lymph glands and bone marrow, the latter appearing to play an important part. In one animal ten months after splenectomy the bone marrow resembled that of leukemia. This change, however, was not constant. Laudenbach (5) (1893) observed in one dog (ten to twelve years of age), with severe anemia, signs of increased blood formation in the marrow 145 days after splenectomy. Ceresole (6) (1895), on the other hand, found in splenectomized rabbits no clearly defined new formation of the marrow. Warthin (1) (1903) states that after splenectomy in the sheep and goat slight lymphoid changes in the fatty marrow occur, but he gives no histological description. Of these changes he says:

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[1] *Jour. Exper. Med.*, 1912, xvi, 363, 376, 758, 769, 780; 1913, xviii, 487, 494, 665.

"The beginning lymphoid changes in the fatty bone marrow in the second and fifth month after splenectomy, are to be regarded as compensatory only for the increased destruction of red blood cells and not for any abrogated splenic function of red cell formation."

Other references may be found to changes in the bone marrow in the presence of diseases of the spleen in man (7) and in experimental anemias of animals with or without splenectomy, but few findings after simple removal of the normal spleen are available. Among the latter are Pugliese's (8) observation that after total splenectomy the bone marrow of the hedgehog becomes filled with giant cells. This change Foa (9) has found not to be characteristic of the rabbit. Vulpius (10) who in 1894 reviewed thoroughly the subject of the surgery and physiology of the spleen, and adds some experimental observations, supports the theory of increased activity of the bone marrow after splenectomy. Winogradow (11) found red marrow in the long bones of a dog 132 days after splenectomy, but yellow marrow was present in two after 517 and 760 days respectively, though one of the latter was slightly streaked with red. Hodenpyl (12) in the description of a case of absence of the spleen in man makes no mention of the bone marrow. Taylor (13) describes the marrow of two splenectomized dogs; that from an animal receiving albumoses by mouth and by hypodermic injection, and killed after nine months, was red; a second splenectomized animal, not receiving albumoses, showed a yellow marrow at the end of one year. Freiberg (14) states that he found red marrow in splenectomized animals, and Gibson (15) notes that in a dog killed five and a half months after being deprived of the spleen, the marrow was apparently in the process of change from yellow to red.

In some of these accounts brief mention is made of the increase of giant cells or of pigmented cells or of the numerical relations between the myelocytes and the white and red cells, but we have been unable to find an adequate account of the histology of the bone marrow after splenectomy based on modern conceptions of the cytology of this tissue. Histological descriptions exist, but they are either brief and fragmentary or are based on views current before the attainment of our present detailed knowledge of the morphology of the cells of the blood.

METHODS.

Our studies are based chiefly on the changes in the marrow of the long bones and particularly in that of the femur. As this marrow in the adult dog is normally fatty, objection may be raised against its use, and to overcome this objection, we attempted to study the marrow of the compact bones. The methods of decalcifying the tissues have, however, in our hands, failed to yield satisfactory

histological preparations. The alternative, the use of film preparations obtained successively at intervals over long periods of observation being impracticable, the study of cover-glass preparations was limited to a single observation at the time of the death of the animal. At the same time, however, in many instances marrow squeezed from the ribs has been obtained in sufficient amount to section and thus to allow a comparison with changes in the fatty marrow. We have, however, depended largely upon the study of sections of the marrow of the long bones and in particular of the femur. We are satisfied, as the result of our study of the marrow form a large number of normal dogs, that this is, after all, the most rational method of studying compensatory changes, for it is unusual even in a definitely fatty marrow not to find numerous centers of blood-forming cells. These may be limited to the periphery of the marrow or be scattered throughout, but whatever their position they afford an excellent starting-point for the study of increased cellular content as well as of changes in the character of the cells. The fatty marrow is of especial value in the study of the late changes, for in well fixed and well stained marrow there can be no doubt about the change from a purely fatty marrow to a red marrow rich in cells. This is so striking as to remove all doubt which exists when one examines the marrow of compact bone, as of the ribs or vertebræ, by either the section or cover-glass method.

We have worked exclusively with the marrow of the middle third of the femur, avoiding the marrow at either end, partly on account of its bony nature, but chiefly because of the occasional occurrence of more or less red marrow at the ends of the shaft. As only adult dogs have been used, we feel that the constant use of the middle portion of the marrow allows fairly comparable results. In removing the marrow half the circumference of the bone through the greater part of its length has been chipped away, and after separating the marrow from the bone and cutting it at either end it has been easily removed as a solid cylinder by gently rolling it on to a piece of filter paper. In carrying these tissues through the process of fixation and imbedding, the filter paper, which is firmly adherent to the marrow through the coagulation of the attached blood, allows the necessary manipulations without injury to the marrow

itself. The routine procedure has been to fix in Zenker's fluid without previous decalcification, imbed in paraffin, and stain with eosin and polychrome methylene blue. Other stains have, however, been used when necessary to bring out certain details.

RESULTS.

It may be stated at the outset that we have found no evidence of an early change in the bone marrow. Splenectomy does not cause, as do successive hemorrhages and hemolytic poisons, a rapid change of fatty marrow to red marrow. This latter change we have produced readily and rapidly in non-splenectomized control animals by the use of specific hemolytic serum and by causing hemorrhage, but we have never seen a frank change from yellow to completely red marrow in the ordinary course of events in the splenectomized animal until many months, usually six or more, had elapsed, and this despite the fact that many of the animals have had, as has been shown in our earlier work, a moderately severe anemia. This anemia has frequently been of as severe degree as that caused by severe successive hemorrhages in the normal dog, but changes in the marrow analogous to those caused by hemorrhage have not been evident in the earlier periods following splenectomy.

In this connection it may be recalled that the anemia of splenectomy in the dog follows a gradual downward course for three to six weeks, the decrease in hemoglobin being relatively more marked than the decrease in red cells, and that an equally gradual repair causes the red cell count and hemoglobin content to approach normal after three to four months or more. At the same time there is a transient initial leucocytosis due chiefly to polymorphonuclear leucocytes, and a more or less constant lymphocytosis with a late eosinophilia. Not infrequently the eosinophils disappear from the circulating blood from the third week until the end of the third month.

We have, therefore, in the course of our studies attempted to determine whether the hyperplasia in the bone marrow after splenectomy is compensatory in the sense of (1) overactivity in red cell formation chiefly, or (2) peculiarly active in the formation of the white cells of the blood, or (3) in the sense of an orderly reproduction of a new marrow with normal activities in the formation of all cells arising within it.

THE NORMAL MARROW OF THE FEMUR OF THE DOG.

In our study of the marrow of both normal and splenectomized animals we have used as a basis for orientation Bunting's (16) conception of erythrogenetic and leucogenetic centers, Muir's (17) descriptions of erythroblastic and leucoblastic reactions, and have received also much aid from Dickson's (18) study of the cytology of marrow. The arrangement described by Bunting is by no means a constant and definite one, but in the masses of marrow cells may be seen groups composed mainly of myeloblasts and surrounded at times by a nearer zone of myelocytes and an outer zone of leucocytes; in other groups with the same center, the outer zone may be made up of nucleated red cells with a still more distant zone of normocytes. We are not convinced that centers for the production exclusively of red cells or of white cells exist, for frequently an intermingling of the two types is seen in one center, but this conception of definite centers is of great assistance in the interpretation of marrow changes.

The study of the marrow of the femur from many normal dogs has led to our recognition of four definite groups of cells: (1) Groups of undifferentiated cells and myelocytes. These lie between fat cells and seem to be in no way connected with blood channels. In all these centers the cells of the connective tissue reticulum are in evidence. (2) Groups of the character described above, but with a peripheral accumulation of cells in which those of the leucocyte series predominate. (3) Groups as in (1), but with a mantle of cells in which those of the erythrocytic series are most in evidence. (4) Groups as in (1), but with an indiscriminate mingling of cells of red and white series.

These groups cannot always be differentiated for not infrequently an indiscriminate mingling of cells obscures the recognition of centers. Moreover, at times may be seen groups composed purely of white cells or of red cells without myelocytic centers. We have, however, found that search for the groupings described greatly facilitates the study of complex marrow pictures and leads readily to a decision as to whether leucoblastic or erythroblastic activity predominates.

In one respect the study of normal marrow has not helped us

greatly. Megakaryocytes and polykaryocytes are so infrequent in the normal fatty marrow that we have no basis, in regard to them, for a comparison with hyperplastic marrow. The same holds true for the large endothelial cells which are phagocytic for red cells and are found so frequently in hyperplastic marrow to contain remnants of red cells and fragments of pigment.

THE MARROW OF SPLENECTOMIZED ANIMALS.

In table I the general results of our observations are presented. The terms "yellow" and "red" refer to the gross appearance, not of the surface of the marrow, but of the cross section. "Slight streaking" and "streaked" refer to an intermingling of yellow and red marrow. A marrow is described as "red" only when it is uniformly so. As will be seen by a comparison of gross and microscopic appearances, a marrow "yellow" to the naked eye may, microscopically, show evidence of beginning hyperplasia. The

TABLE I.

Hyperplasia of the Marrow of the Femur after Splenectomy.

Dog No.	Period after splenectomy.	Gross appearance.	Microscopic change.
50	24 dys.	Yellow	Slight.
23	39 dys.	Yellow	None.
21	40 dys.	Yellow	Slight.
86	42 dys.	Yellow	Slight.
79	60 dys.	Yellow	Slight.
82	63 dys.	Slight streaking	Slight.
17	84 dys.	Yellow	Slight.
10	6 mos.	Red	Complete.
39	7 mos.	Red	Complete.
32	8 mos.	Yellow	Slight.
44	$9\frac{1}{2}$ mos.	Yellow	None.
41	10 mos.	Yellow	None.
24	1 yr.	Red	Complete.
59	$1\frac{1}{2}$ yrs.	Red	Complete.
57	$1\frac{1}{2}$ yrs.	Streaked	Partial.
33	$1\frac{3}{4}$ yrs.	Red	Almost complete.
51	$1\frac{5}{6}$ yrs.	Yellow	Slight.

early changes are indicated by the word "slight." The word "complete" indicates that only an occasional fat cell is seen microscopically. "Almost complete" means that fat cells are present in the proportion of one part to nine of marrow cells, in the surface area of sections studied. Several purely fatty marrows represent-

ing periods between five and twenty-four days after splenectomy are not included in the table.

The bone marrows representing the earlier periods of splenectomy, in that they show practically no changes, may be dismissed briefly. This is true of a series from animals killed at various intervals from five days to three months. Some of these marrows cannot be distinguished from those of the normal dog. In others, slight replacement of fatty tissue is seen. Thus, one representing the twenty-fourth day shows here and there between the fat cells single rows of blood-forming cells with now and then clumps of ten to thirty or more. These areas are neither purely erythrogenetic or leucogenetic, though in some of the groups with an older type of cells there is a predominance of polynucleated cells. The endothelial cells of the reticulum not infrequently contain large masses of old blood pigment.

Another, representing the fortieth day, presents practically the same appearance with a tendency, however, to greater erythrogenesis. On the other hand, a thirty-nine day dog shows a simple fatty marrow with no evidence of active blood formation. Three other marrows of this period, however, show already the early stages of hyperplasia. In one of these (forty-second day) showing a slight general hyperplasia, both types of cell groups can occasionally be isolated, but usually the groups are mixed. Greater numbers of eosinophil cells, both myelocytic and polymorphonuclear, are present than have been evident in earlier periods. A number of cells throughout the section correspond to Longcope's (19) small lymphocytes and a smaller number to Longcope's large lymphocytes. The small lymphocytes are not, however, in pure groups. The picture as a whole is more one of leucogenesis than of erythrogenesis. Very few giant cells are seen and only occasional phagocytes. Polymorphonuclear leucocytes are abundant.

In another marrow of the sixty-third day, a moderate peripheral hyperplasia of mixed type is present. Marked congestion is evident between the fat cells, and in places near the periphery there is hyperplasia; in some places the erythrocytes appear to be outside the vessel, forming distinct hemorrhages. A few phagocytes are present, but giant cells are rare. Polymorphonuclears are frequent and of

mature development. At the periphery erythrogenesis seems to predominate over leucogenesis. Eosinophils and lymphoid cells are not conspicuous.

A marrow of the sixtieth day shows less hyperplasia, but leucocytic reaction is more evident, though erythrogenesis is active. Scattered throughout the section are many small lymphocytes, but nowhere are these seen in solid clumps. Numerous deposits of pigment are seen.

Again on the eighty-fourth day an essentially fatty marrow shows a narrow cellular strip at the periphery in which erythrogenesis is active. Here and there leucogenesis predominates, but in the main the process is erythrogenetic. A few nucleated red cells of the megaloblastic type are found, but the more mature normoblasts are more abundant. In some centers radiating lines of four or five normoblasts are seen. Few giant cells are present.

The changes of the fourth and fifth months after splenectomy are not represented in this study. Well marked hyperplasia is, however, present in bone marrow representing periods of 6, 7, 12, 17, 18, and 20 months after splenectomy. On the other hand, two marrows representing respectively nine and a half and ten months show no departure from the normal fatty marrow, and in a third (eight months) only slight hyperplasia is evident. In the latter are areas composed almost entirely of cells of the myelocyte or premyelocyte type with some evidence of the formation of both red cells and polymorphonuclear leucocytes. The picture suggests a proliferation of the primitive cells of the marrow without, however, a very active function on their part. With evidence of well marked hyperplasia at six and seven months and after a year and a year and a half, it is impossible to explain its failure in these three animals representing the 8th, 9th, and 10th months respectively.

The best opportunity of studying the late changes is presented by material from six animals, representing the period from six to twenty months, in all of which the fatty marrow of the femur was transformed entirely or in large part into red marrow. The histological picture of each of these will be given in detail.

Dog 10.—Splenectomized May 20, 1913. Before operation the red cells numbered 6,910,000 and the hemoglobin was 105. The severest anemia was about

July 21; red cells 4,240,000, hemoglobin 62. On Sept. 11 the figures were 5,220,000 and 92. Later the animal became pregnant and anemia recurred, the picture on Nov. 18 being red cells 4,410,000, hemoglobin 78 per cent. On Nov. 24 the animal was chloroformed. At autopsy the medulla of both femurs presented a deep red marrow.

Histological Examination.—A uniformly cellular tissue is seen with occasionally a fat space here and there at the periphery. For the most part this marrow is as definitely cellular as is, for example, a lymph node or the spleen, and indeed it has the appearance of the pulp of the latter organ in the new born puppy. In this cellular mass, which at first appears to present a hopeless confusion of cells, it is not difficult to resolve the cells into more or less distinct proliferating centers. The arrangement is by no means a definite one, but in the patchwork of cells one sees groups which correspond to Bunting's description. In speaking of these centers we shall refer to them as erythrogenetic or leucogenetic, according to whether red cells or polymorphonuclear leucocytes predominate in the mass of cells surrounding the center in question. We have made no attempt to distinguish in these centers, which may include 6 to 10 or 20 to 30 cells, between the finely granular neutrophil myelocyte and the non-granular basophil cell from which it is supposed to arise. In these centers mitotic figures may occasionally be seen but only after prolonged search. It is also in these centers that old blood pigment, which is quite abundant in this marrow, is deposited; its deposition in the loose vascular tissue elsewhere has not been observed. The erythrocytic centers appear to be more active than the leucogenetic. This impression is based on the fact that about a mass of myelocytes composed of twelve to fifteen cells may be seen twenty-five to thirty nucleated red cells and a small number of normocytes, while about the leucogenetic centers comparatively few leucocytes are seen. The red cells in question vary in size and show changes from the megaloblast to the normocyte. It is not to be supposed that about erythrocytic centers no leucocytes occur. A few are always present; for example, among the twenty-five to thirty cells mentioned above, eight polymorphonuclear leucocytes could be clearly distinguished. Sometimes on one side of a center nucleated erythrocytes may be grouped, and on the other leucocytes with little intermingling. This suggests simultaneous formation of the two cells in one cell center. When this occurs the number of red cells is always greater than the number of leucocytes, in the proportion of 30 to 8. All through the section are lymphoid cells, usually single and of the small variety. Giant cells are frequent and a few show inclusions of polymorphonuclear leucocytes. Cells containing such inclusions have a broad homogeneous gray staining protoplasm suggesting necrosis. There is considerable pigment, but not many phagocytic endothelial cells are seen. Normoblasts are seen free in the capillaries. Smears from the marrow of the ribs show active erythrogenesis, and, on the whole, much the same cellular picture as the marrow just described. In the rib marrow a considerable number of eosinophils, chiefly polymorphonuclears, are also seen. In connection with the activity in the formation of red cells shown by the marrow, it is significant that the blood count six days before death was 4,100,000, and the hemoglobin 78 per cent. On Sept. 11, four months after splenectomy, the figures were 5,240,000 and 92. In other words, despite the hyperplasia of the bone marrow

the animal exhibited a late anemia, two and a half months after recovery from the initial anemia, following splenectomy. This may have been due to the drain occasioned by the intervening pregnancy,—an unfortunate complication from the point of view of the study of the blood. The fact remains, however, that the marrow is actively forming normal red cells. The anemia was, therefore, not due to insufficient erythrogenesis in the marrow.

Dog 39.—On Apr. 7, 1913, before splenectomy, the red cells numbered 6,528,-000, and hemoglobin was 110 per cent. The lowest point of anemia was reached on June 3, the red cells numbering at that time 3,650,000; the hemoglobin was 62 per cent. By July 7 the blood picture had improved (red cells 5,080,000, hemoglobin 88 per cent.), but on Sept. 11, a late recrudescence of the anemia gave red cells 4,040,000 and hemoglobin 68 per cent. The animal was killed on Nov. 15. The bone marrow of the femur was of a definite red color. The anemia did not affect the general nutrition of the animal. On Apr. 7 the weight was 12,800 gm.; on Nov. 15 it was 13,950, and the adipose tissue was abundant.

This animal, representing practically the same period after splenectomy and the same changes in the blood, presents very much the same picture in the marrow. Of minor importance is the fact that the marrow is not so cellular, the proportion of cells to fat being in the ratio of about 3 to 2; also the myelocytic centers are not so pronounced, but in other respects the marrow is the same. Many giant cells are present but lymphoid cells are rare. The formation of red cells and leucocytes is perhaps not so rapid; that is, the numbers about any one center are not so great but, on the other hand, the activity of the marrow in connection with the former is sufficient to exclude the possibility of the bone marrow being responsible for the later development of anemia.

Dog 24.—This animal was splenectomized on Feb. 10, 1912, and was used for the injection of hemolytic immune serum on Mar. 20 and again on Apr. 7. On June 28 it had recovered from the anemia then produced (red cells 5,650,000, hemoglobin 89 per cent.), and on July 15 it was treated with sodium oleate. On Sept. 26 the red cell count was 5,780,000, and hemoglobin 90 per cent. On Feb. 19 the blood picture had improved (red cells 6,048,000, hemoglobin 110 per cent.), and at this time hemolytic serum was again injected. The animal was chloroformed on Mar. 4, 1913. The lapse of time since splenectomy was, therefore, thirteen months. At autopsy a red marrow was found.

Owing to the use of various hemolytic poisons, the bone marrow of this animal may have been influenced by other factors than the absence of the spleen. The histological picture, however, is so in accord with the marrow of simple splenectomy that, with this explanation, it is included in the series.

Histological Examination.—The marrow is uniformly solid with no fat spaces visible in any of the sections examined. It does not, however, appear to be as cellular as the marrow of dogs 10 and 39. This difference is caused by a greater congestion and distention of the blood vessels, a slight increase in the reticulum, and a lessened tendency of the myelocytic tissue to be grouped in large centers. Erythroblastic centers are very prominent and very active; leucogenetic centers, on the other hand, are made out with difficulty. Lymphoid elements are rare. Many cells of the myelocytic type are seen with coarse

basic granules and short threads in the nucleus, and with little or no protoplasm. In close relation to these are sometimes seen degenerated mitoses, but whether all these masses can be so interpreted is not clear. These degenerative changes are doubtless the result of the last injection of hemolytic serum.

Dog 59.—On July 24, 1913, this animal was splenectomized and used for the study of the progressive anemia following this procedure. On Dec. 7, 1912, the highest point of recovery was reached (red cells 5,250,000, hemoglobin 105 per cent.). Continued observation showed a slight decline to 5,200,000 red cells and 86 per cent. of hemoglobin on May 21, 1913, on which date the animal was used in an experiment with sodium oleate. From the moderate anemia caused at this time, the animal quickly recovered, the blood examination on June 9 showing 5,050,000 red cells and 86 per cent. hemoglobin, the condition slightly improving as to hemoglobin content until Nov. 18, 1913, when red cells were 5,100,000 and hemoglobin 101 per cent. The animal was chloroformed on Nov. 24. At autopsy the bone marrow of the femur was soft, succulent, and dark red in color. In connection with the general condition of this animal it is of interest to note that in the last seven months its weight increased from 10,450 to 12,580 gm., and that adipose tissue was very abundant. The administration of sodium oleate introduces a possible disturbing factor, but as this was given six months before death, it is not considered, in view of our other results, a serious matter.

Histological Examination.—The marrow shows some fat cells, the proportion of marrow cells to fat being about 10:1. Nothing new is presented. Leucogenesis and erythrogenesis proceed at about equal rate, the latter being a little more active. Mitotic figures are seen not infrequently, but the type of cell in which they occur is not always evident. Myeloblasts seem to be more abundant than usual. Giant cells are fairly abundant, but lymphoid cells are rare.

Dog 57.—On June 23, 1912, the blood of this dog contained 5,350,000 red cells per cubic millimeter, and 98 per cent. hemoglobin. On July 2 the spleen was removed. The resulting anemia reached its lowest point (red cells 2,970,000, hemoglobin 50 per cent.) on Aug. 5. On Oct. 24, two days after the blood count showed 5,240,000 red cells and 90 per cent. hemoglobin, the animal received sodium oleate intravenously; a very slight anemia (fall in hemoglobin to 62 per cent., but no change in red cells) resulted. In Jan., 1913, the red cells were 5,206,000, hemoglobin 110 per cent., and with slight variations this higher level was maintained, accompanied by an increase in body-weight, until Dec. 12, 1913, when the animal was chloroformed. At autopsy the animal was found to have a large amount of adipose tissue; the bone marrow of the femur was definitely reddish in color with faint yellowish streaks. As the sodium oleate given four months after splenectomy and fourteen months before death produced only a slight transient change, we consider that the bone marrow represents the effect of splenectomy only.

Histological Examination.—The relation of the fat to cells is about 1 : 1; otherwise nothing new is seen. The marrow is very active, leucogenesis and erythrogenesis being equally prominent. Phagocytic cells and masses of old blood pigment are quite numerous, as are also giant cells. More abundant than

in other marrows are eosinophils of the myelocytic type. Lymphoid cells are not conspicuous.

Dog 33.—This animal was splenectomized on May 14, 1912, the blood examination on the previous day showing 4,950,000 red cells and 85 per cent. hemoglobin. The anemia following splenectomy reached its lowest point on June 28 (red cells 3,550,000, hemoglobin 52 per cent.). On Sept. 20 the red cells had risen to 5,490,000 and hemoglobin to 95 per cent. In Nov., 1913, the animal passed successfully through pregnancy. In Jan., 1914, as the animal had developed mange, it was chloroformed. The blood examination on the preceding day was red cells 4,480,000, hemoglobin 70 per cent. At autopsy the bone marrow of the femur was deep red in color. It should be stated that one and two months before splenectomy the animal had received injections of hemolytic serum. From our studies of the effect of hemolytic serum in the normal dog, we do not believe that these injections, nearly two years before death, are in any way responsible for the hyperplasia of the marrow.

Histological Examination.—This marrow differs in no way from those of dogs 57 and 59 described above.

Dog 51.—The spleen was removed on May 31, 1912, and on June 26 hemolytic serum was administered. From the anemia thus produced the animal made a slow recovery, but after 200 days the blood examination showed 6,200,000 red cells and 110 per cent. hemoglobin, as compared with 6,210,000 red cells and 100 per cent. hemoglobin before splenectomy. On Mar. 26, 1914, when the animal was chloroformed, its weight was 9,750 gm. as compared with 8,270 gm. at the time of splenectomy, and 8,120 when hemolytic serum was administered. The notes made at the autopsy refer to the large amount of adipose tissue, the normal appearance of the lymph nodes, the absence of supernumerary spleens, and the presence in the long bones of a distinctly yellow fatty marrow.

Histological Examination.—The marrow shows a very slight hyperplasia with large numbers of leucocytes and deposits of blood pigment.

DISCUSSION.

In view of the slight changes seen in the bone marrow during the early periods after splenectomy, it appears that neither during the period of anemia and consequent repair nor in the period of hyperplasia of bone marrow are nucleated or other irregular forms of red cells found frequently in the peripheral blood. Careful differential counts of three dogs at regularly spaced intervals for 138 days failed to reveal in two the presence of nucleated red cells, and in one they were found only five times, the largest number seen in one count being three. In a large number of other animals in which differential counts were made at irregular intervals, changes in the red cells have been found very rarely; in one dog five weeks after splenectomy five normoblasts and two megaloblasts were

found (in 100 cells) with evidence of poikilocytosis and polychromatophilia, and a week after one normoblast and one megaloblast. These findings correspond to the first days of beginning repair, the red cells and hemoglobin having a few days before reached the lowest level observed during the experiment; hemoglobin 50 per cent., red cells 2,970,000. In another, two months after splenectomy, again at the stage of beginning repair (hemoglobin 92, red blood cells 3,650,000) five nucleated red cells were found, and a polychromatophilia was evident. In no instance did these findings persist for any length of time. If they have any significance it is that they indicate the period of beginning repair.

It is difficult to bring the changes in the bone marrow into relation with the changes in the peripheral blood. If the hyperplasia of the bone marrow is compensatory to increased blood destruction, or decreased blood formation, one would expect definite hyperplasia to be present in the earlier period, during the first three months after splenectomy, at a time when the anemia is evident and repair is taking place, and not after six months to a year or a year and a half when the blood picture is normal. It is true that in two of the animals (dogs 10 and 39) a late recrudescence of anemia occurred and the marrows of these animals were obtained during this period, but this was not the case in other animals of the series and is not characteristic of the late periods after splenectomy. It is therefore impossible, on account of the late development of hyperplasia in the marrow, to explain its occurrence as compensatory to the anemia following splenectomy.

Likewise we cannot accept Warthin's theory based upon his study of sheep and goats. In these animals Warthin found hyperplasia of the marrow to occur several months after splenectomy and to be associated with evidence of increased destruction of red blood cells in the lymph and hemolymph nodes. This destruction, greater than that in the primitive spleen, is responsible, he believes, for the anemia following splenectomy, and this is in turn compensated by increased activity in the bone marrow. We have found little to support this theory in our studies of the dog. The lymph nodes, as well as the endothelial cells of the liver, as we have shown elsewhere (20), are indeed more active after splenectomy than in the

normal animal in the phagocytosis and destruction of red cells, and this is very evident when large numbers of red cells are injured, as by the administration of a hemolytic poison; but in the ordinary course of events, after splenectomy, the lymph nodes present no evidence of excessive blood destruction. An occasional cell containing one or two red cells may be seen and small amounts of old blood pigment are occasionally demonstrable, but of excessive hemolysis there is no evidence. Microchemical tests for iron in the lymph nodes of fifteen splenectomized dogs showed a considerable amount of iron in three, slight amounts in five, and none in six. The animals examined represented periods of eleven days to twenty-two months after splenectomy. In the lymph nodes of eight normal animals similarly examined, moderate amounts of iron were found once, slight amounts three times, and in four, none. It is evident, therefore, that in the dog the iron content of the lymph nodes after splenectomy differs little from normal. The liver likewise shows no increased deposition of iron. Of fourteen livers from splenectomized dogs, four showed slight depositions of iron in the Kupffer cells, while ten showed none. At the same time the livers of six normal dogs were similarly examined; in three slight deposits of iron were found, and in three none. For this reason, and because the anemia is not persistent and progressive we cannot support the theory that the hyperplasia of the marrow is compensatory to abnormal blood destruction in the lymph nodes.

Another possible explanation is that the bone marrow, in the absence of the spleen, is concerned in the storing and utilization of iron. There is no doubt that, in the intact animal, iron set free in the dissolution of red cells is stored in the spleen. After splenectomy a readjustment in the storage of iron takes place, and there is some evidence that for a short time after the removal of the spleen iron is lost to the body. It is possibly this disturbance of iron utilization that is responsible for the early transient anemia. Our investigations[2] show, however, that this disturbance of iron utilization is transient and that after a few weeks the elimination of iron in the splenectomized animal differs in no way from the process in the normal animal. This suggests naturally that the storage of

[2] To be presented in detail in a future communication.

iron in the absence of the spleen is taken over by other tissues. As microchemical tests for iron showed no definite increase of iron in the lymph nodes and liver it seemed probable that the bone marrow might be the chief depot of iron storage. Such a view was supported by the fact that all hyperplastic bone marrows contain large amounts of altered blood pigment, sometimes free, but occurring, for the most part, in large phagocytic cells. The activity of these phagocytic cells presumably in transforming the iron of old blood pigment in order that it may be reutilized by the red cells, might, it was plausible to suppose, stimulate the other functions of the bone marrow, that is, the erythrogenetic and leucogenetic functions and cause eventually a replacement of the fatty marrow by a very cellular red marrow.

In order to prove this hypothesis it was necessary to obtain some idea of the iron content of these marrows. Direct chemical analysis was out of the question on account of the small amount of material available and the variations in blood and bone content of different marrows. We therefore made a comparative study based on the use of the microchemical reaction for iron. This demonstrated at once that all red marrows in our series have a large content of iron, and that fatty marrows contain very little or no iron. On the other hand, when the marrows of non-splenectomized dogs rendered hyperplastic by anemia or infection were examined it was found that these also had a large iron content. Thus in a group of 17 non-splenectomized dogs iron was present in the marrow in large amounts in 4, in moderate amounts in 2, in small amounts in 4, and in 7 none was found. On the other hand, in 27 splenectomized dogs, iron was present in large amounts in 10, in moderate amounts in 3, in small amounts in 4, and absent in 10.

In both groups the amount of iron was in direct proportion to the degree of hyperplasia. These observations point therefore to the conclusion that a red marrow is always rich in iron, but it is impossible to say whether the cellular hyperplasia or the iron deposition is primary. Under the circumstances, it is also impossible to conclude that the late hyperplasia of marrow following splenectomy is an attempt to conserve iron. Moreover, the irregularity of our results as shown by the failure of hyperplasia in four animals,

representing respectively the 8th, 9th, 10th, and 22d months after splenectomy, prevents in the present state of our knowledge an adequate explanation of the cause of the transformation from yellow to red marrow.

The divergent results in this study are characteristic of all phases of experimental work on the spleen and doubtless are to be explained by the fact that removing the spleen takes away only one organ of a system composed of liver, spleen, lymph nodes, and bone marrow, and that the interrelations which exist in this system may or may not under varying circumstances bring into play compensations of the greatest importance in determining the degree of blood destruction or regeneration and therefore the degree of change in the bone marrow.

A search of the literature of splenectomy in man, although it reveals evidence of the occurrence of red marrow in various forms of splenic anemia (7), offers little of importance concerning the changes which occur in the bone marrow after removal of the normal spleen. Several references (21) are made to the occurrence of pain in the long bones after splenectomy, and by some this has been assumed to be evidence of hyperplasia within the rigid bony canal. The only note of the direct examination of the bone marrow after splenectomy is that of Riegner (22), who found active proliferation of the marrow of the femur in a man whose leg was amputated for gangrene four weeks after splenectomy for trauma. It is therefore impossible, on account of this paucity of data concerning the changes in man, to bring them into relation with our experimental results.

CONCLUSIONS.

Splenectomy in the dog causes, as a rule, a transformation of the fatty marrow of the long bones to a richly cellular red marrow.

During the early periods, one to three months, the change in the marrow is slight and either focal or peripheral; after six to twenty months the replacement of fat by marrow cells is complete or nearly so. Exceptions were, however, seen in four animals representing the 8th, 9th, 10th, and 22d months, respectively. The evidence at hand does not support the theory that this hyperplasia is compen-

satory either to the anemia caused by splenectomy or to an increased hemolysis in the lymph nodes. It is possible that it may be a concomitant of the activity of the bone marrow in taking over, in the absence of the spleen, the function of storing and elaborating the iron of old blood pigment for future utilization by new red cells, but our studies do not fully support this view.

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THE MECHANISM OF ANAPHYLATOXIN FORMATION.

Studies on Ferment Action. XV.*

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The attention that the problems of anaphylaxis and of anaphylatoxin formation have received during the past few years has led to two antagonistic conceptions as to the nature of the mechanism of intoxication.

Friedberger (1) believes that the phenomena are due to an intoxication by protein split products, placing the matrix of the poison in the foreign protein, or antigen, introduced. Doerr (2) regards them as physical and as resulting from colloidal changes in the serum, and denies the possibility of enzymotic protein cleavage as having any direct bearing on the process of intoxication. Keysser and Wassermann (3), Ritz and Sachs (4), and Bordet (5) are more or less committed to the physical theory, although they do not consider it necessary to go as far as Doerr in denying the splitting of the proteins, merely placing the matrix of the toxin in the homologous serum, without definite agreement as to the nature of the colloidal changes that are supposed to occur.

The production of the so called anaphylatoxin *in vitro* by Friedemann (6) and by Friedberger and Mita (1), by the interaction of complement, antigen, and amboceptor, has been greatly simplified. Friedberger himself found the amboceptor superfluous. Almost any adsorbing substance has been used as an antigen; such as kaolin (Keysser and Wassermann (3)), agar (Besredka and Ströbel (7), and Bordet (5)), "*Kieselguhr*" (Doerr (2)), starch (Nathan (8)), waxes and fats from bacilli (Leschke (9)), besides the true antigens, which latter need not be specific, for they are still effective after boiling. The experiments of Doerr and Russ (2), Kraus (10), Seitz (11), Aronson (12), and Friedberger have shown that complement is not essential, for toxic sera can be produced from inactivated serum, although not with the same regularity as from active serum.

If, then, the antigen-antibody hypothesis has failed to explain the accumulated facts, how are we to interpret the toxicity of the so called anaphylatoxins? The evidence still seems strong that the

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toxic substances are cleavage products of proteins, or, at any rate, proteins so altered that they are capable of being split in the organism; for Friedberger does not claim that the entire toxic effect of anaphylatoxin is due to preformed split products. The arguments made against the protein-intoxication conception, such as the time element and the minute amount of substance necessary, are not convincing, for we know that ferment action may be very rapid; and as far as the quantity of substrate is concerned the argument fails if we place the matrix of the poison in the serum proteins themselves. There is some reason to believe, however, that if ferment action is the basis of anaphylactic shock, these ferments may have a much wider range of action than merely on the introduced protein.

Doerr (2) assumes that guinea pig serum is normally toxic, but that the toxicity is held in abeyance by some unknown substance which is adsorbed during the anaphylatoxin formation. Ritz and Sachs (4) suggest that during adsorption alteration occurs in the serum resulting in the formation of toxic substances. In a measure, both of these views are probably correct, although in neither of the papers in which they are put forward are there to be found suggestions as to the nature of the adsorbed substances. Doerr calls attention to the marked changes in coagulation as manifestations of the intoxication and suggests a causal connection. We, too, feel certain that this phase warrants a more detailed study.

Granting tentatively that anaphylaxis and anaphylatoxin intoxications are due to protein split products, we come to a consideration of the matrix. That the antigen used in producing anaphylatoxin is not split, has been convincingly shown by Donati (13) who noted that organisms so used retained their antigenic properties; while Neufeld and Dold (14) observed that living bacteria produced anaphylatoxin more easily than killed organisms. The fact that the same antigen can be used repeatedly would also tend to confirm this view. The demonstration of dialyzable split products occurring in anaphylatoxin (Pfeiffer and Mita (15)) gives no clew to the origin of the cleavage products; and inasmuch as we have recently (16) shown that normal serum will autolyze rapidly when the antiferments are lowered or removed, there is reason to believe that small amounts of split products may occur in anaphylatoxin. Total non-coagulable nitrogen determinations made according to the method of Folin show, however, that whatever splitting takes place in the usual methods of anaphylatoxin formation is very small. The production of anaphylatoxins by means of kaolin, agar, starch, waxes, and infusorial earth leaves no room for doubt that the matrix must reside in the serum.

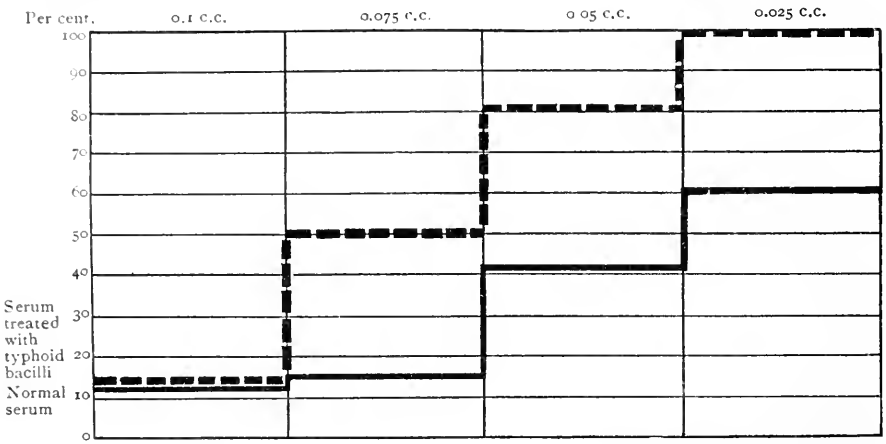
In a recent paper (16) we have demonstrated the lipoidal nature of serum antitrypsin and have shown that the inhibition is due to

unsaturated fatty acids and esters (17). We have also shown (18) that by extracting the serum with chloroform or ether, thereby removing the antitrypsin, or by saturating the free bonds with iodin, or by acidulating and filtering the serum, the serum becomes highly toxic, this toxicity depending on three factors; *i. e.*, changes in coagulation, exposure of serum proteins, and autolysis *in vitro* of the exposed proteins. Under these conditions the serum becomes from five to ten times as toxic as the ordinary anaphylatoxin, the exact minimum lethal dose depending on the amount of lipoids removed and the state of autolysis. The increase in toxicity is accompanied by a constant increase in the total non-coagulable nitrogen as determined by the Folin method, until a period of digestion is reached when the serum is no longer toxic. The mere inactivation of serum antitrypsin by heat or acid is not sufficient to secure a toxic serum, possibly because the lipoids remain in the serum and are rendered active again on reinjection. From the serotoxin which we have prepared we have isolated toxic split products, thermostabile, and lethal in doses of about 0.01 of a cubic centimeter per gram weight of guinea pig when made up to the original serum volume.

The fatty acids and their salts are easily adsorbable substances (19), and we might therefore expect that under proper quantitative conditions it would be possible to adsorb these protective lipoids from serum without affecting the other constituents. We have therefore examined the effect of the various adsorbing substances which have been found to produce typical anaphylatoxin, on the serum antitrypsin of fresh guinea pig serum and other sera. Our experiments have been made by incubating the mixture for three hours and then testing the antitryptic effects directly or after permitting the mixture to remain over night in the ice chest. In the charts illustrating these experiments the antitryptic value of the untreated serum is shown in the heavy black lines. Inasmuch as the ordinary Fuld-Gross technique for antitrypsin determination leads to serious errors in the reading, we have, in every instance, determined the total nitrogen digestion by the method of Folin, which we have described in our former papers.

EXPERIMENTS WITH TYPHOID ANAPHYLATOXIN.

Fresh guinea pig serum was mixed with typhoid bacilli in the proportion of two cubic centimeters of serum to one agar slant of bacilli and the mixture was incubated for three hours. The mixture was then placed in the ice chest over night. As will be seen from text-figure 1, there was a marked decrease in the antitryptic value of the serum so treated. There was no increase in the non-coagulable nitrogen of the serum. A portion of the mixture was



TEXT-FIG. 1. Adsorption of serum antitrypsin from guinea pig serum by typhoid bacilli.

centrifuged until it became clear, and was then injected intravenously into guinea pigs to determine its toxicity. Two cubic centimeters caused immediate death of a guinea pig weighing 210 grams, with all the evidences of anaphylaxis. The experiments have been repeated several times with similar results.

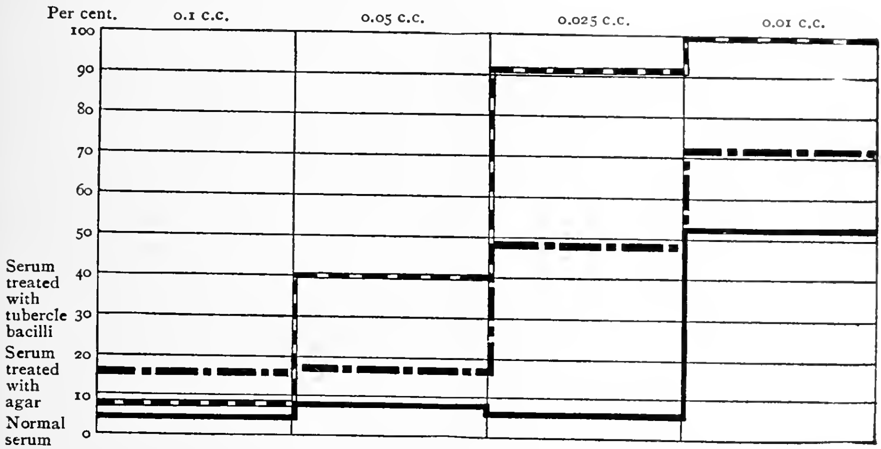
EXPERIMENT WITH AGAR AND TUBERCLE BACILLI ANAPHYLATOXIN.

A similar experiment was made with dried tubercle bacilli and also with a small amount of solid agar. As will be seen from text-figure 2, tubercle bacilli have somewhat, and the agar has greatly lessened the serum antitrypsin. It is interesting to recall that the production of anaphylatoxin from tubercle bacilli has always been somewhat difficult, and that Shibayama (20) found

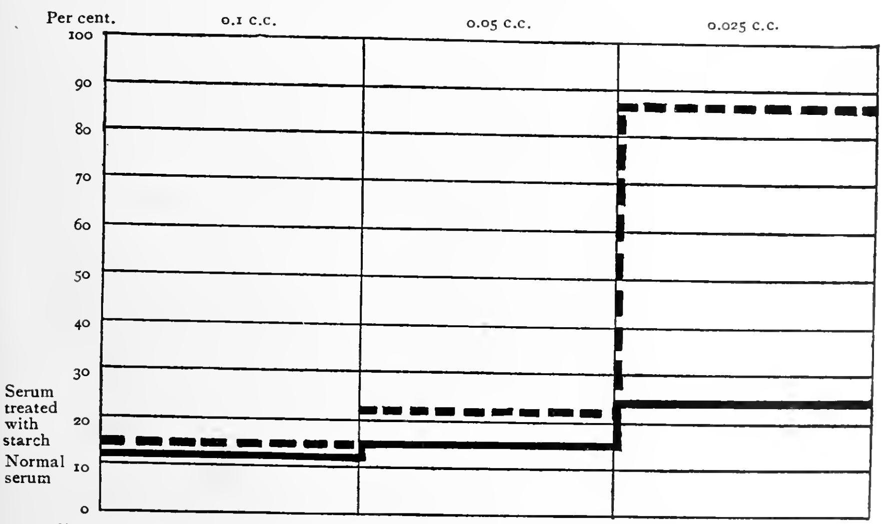
that by extracting the fats from the bacilli the result was much more constant.

ANAPHYLATOXIN FROM STARCH.

The next experiment was made with starch as an adsorbent. The data from which text-figure 3 has been made will be found in protocol I. In this experiment a large amount of starch paste



TEXT-FIG. 2. Adsorption of serum antitrypsin by agar and tubercle bacilli.



TEXT-FIG. 3. Adsorption of serum antitrypsin by starch.

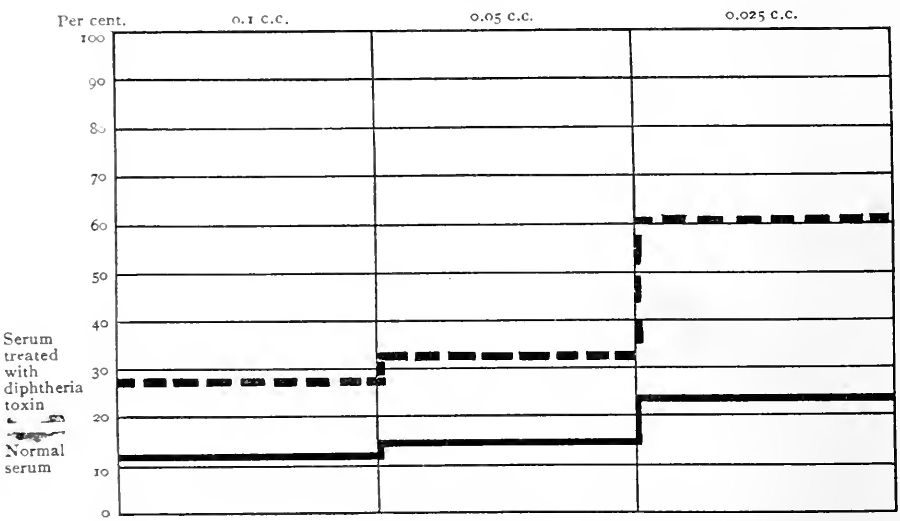
adsorbed less of the antitrypsin than a smaller amount, which is in accord with the actual toxic effects observed by Nathan (8).

The following experiment illustrates the method that we have used in this series of experiments.

2 c.c. of fresh guinea pig serum were mixed with each of the following substances:

1 c.c. of diphtheria toxin (minimum lethal dose = 0.0025 c.c.).
0.1 c.c. of diphtheria toxin (minimum lethal dose = 0.0025 c.c.).
0.2 gm. of kaolin.
0.05 gm. of kaolin.
0.1 c.c. of starch paste.
0.05 c.c. of starch paste.
0.25 gm. of agar (semisolid) active serum.
0.25 gm. of agar (semisolid) inactivated serum.

The mixtures were incubated for three hours, placed in the ice chest over night, and the next morning each tube was diluted one tenth to twenty cubic centimeters with normal salt solution. The



TEXT-FIG. 4. Effect of diphtheria toxin on serum antitrypsin.

kaolin, starch, and agar sera were then centrifuged, and the supernatant serum was mixed with trypsin in the proportion shown in protocol I, incubated for thirty minutes, and two cubic centimeters of 1 per cent. casein solution were then added. After digestion was complete; the undigested casein and serum proteins were pre-

PROTOCOL I.

No. of tube.	1 per cent. casein solution.	Trypsin.	Serum 1:10 + diphtheria toxin.		Serum 1:10 + kaolin.		Serum 1:10 + starch.		Serum 1:10 + agar 0.25 gm.		Normal guinea pig serum 1:10.	Total nitrogen digestion.	Digestion.	
			1 c.c. toxin.	0.1 c.c. toxin.	0.2 gm. kaolin.	0.05 gm. kaolin.	0.1 c.c. starch.	0.05 c.c. starch.	Active.	Inactive 54° C.				
1	2 c.c.	0.0 c.c.										Trace	0%	
2	2 c.c.	0.2 c.c.										1.66 mg.	100%	
3	2 c.c.	0.2 c.c.	1.0 c.c.									0.45 mg.	27%	Diphtheria toxin.
4	2 c.c.	0.2 c.c.	0.5 c.c.									0.55 mg.	37%	
5	2 c.c.	0.2 c.c.	0.25 c.c.									1.0 mg.	60%	
6	2 c.c.	0.2 c.c.		1.0 c.c.								0.26 mg.	15%	
7	2 c.c.	0.2 c.c.		0.5 c.c.								0.3 mg.	17%	
8	2 c.c.	0.2 c.c.		0.25 c.c.								0.8 mg.	47%	
9	2 c.c.	0.2 c.c.			1.0 c.c.							0.2 mg.	12%	Kaolin.
10	2 c.c.	0.2 c.c.			0.5 c.c.							0.2 mg.	12%	
11	2 c.c.	0.2 c.c.			0.25 c.c.							0.3 mg.	17%	
12	2 c.c.	0.2 c.c.				1.0 c.c.						0.3 mg.	17%	
13	2 c.c.	0.2 c.c.				0.5 c.c.						0.74 mg.	44%	
14	2 c.c.	0.2 c.c.				0.25 c.c.						0.93 mg.	56%	
15	2 c.c.	0.2 c.c.					1.0 c.c.					0.13 mg.	8%	Starch.
16	2 c.c.	0.2 c.c.					0.5 c.c.					0.48 mg.	28%	
17	2 c.c.	0.2 c.c.					0.25 c.c.					0.7 mg.	42%	
18	2 c.c.	0.2 c.c.						1.0 c.c.				0.26 mg.	15%	
19	2 c.c.	0.2 c.c.						0.5 c.c.				0.36 mg.	21%	
20	2 c.c.	0.2 c.c.						0.25 c.c.				1.43 mg.	86%	
21	2 c.c.	0.2 c.c.							1.0 c.c.			Lost	Lost	Agar.
22	2 c.c.	0.2 c.c.							0.5 c.c.			0.35 mg.	20%	
23	2 c.c.	0.2 c.c.							0.25 c.c.			0.83 mg.	50%	
24	2 c.c.	0.2 c.c.								1.0 c.c.		0.25 mg.	15%	
25	2 c.c.	0.2 c.c.								0.5 c.c.		0.37 mg.	21%	
26	2 c.c.	0.2 c.c.								0.25 c.c.		1.25 mg.	75%	
27	2 c.c.	0.2 c.c.									1.0 c.c.	0.21 mg.	12%	Normal.
28	2 c.c.	0.2 c.c.									0.5 c.c.	0.24 mg.	15%	
29	2 c.c.	0.2 c.c.									0.25 c.c.	0.4 mg.	24%	
30	0 c.c.	0.0 c.c.	0.05 c.c. diphtheria toxin									0.1 mg.		

All tubes were made up to 5 c.c. with normal saline solution.

cipitated by salt and acid, boiled, filtered through kaolinized hard paper filters, and nitrogen determinations made on the filtrate. The nitrogen determined for each tube in reference to the total digestion of the casein gives the exact percentage of digestion.

Text-figure 4 shows the effect of diphtheria toxin on the serum antitrypsin and illustrates the digestion in tubes 3, 4, and 5 of protocol I. The amount of non-coagulable nitrogen in 0.05 of a cubic centimeter of diphtheria toxin (the largest amount used) was found to be 0.1 of a milligram.

It will be noted that kaolin had not affected the antitrypsin when the larger amount was used, but the smaller amount (0.05 of a gram) had adsorbed a large amount. Our experience has been that agar and starch and bacteria are more effective, as a rule, than kaolin, which is only effective in a certain definite quantitative relation. In the series with agar the inactive serum also shows adsorption of the antitrypsin. We have found it on the whole more difficult to secure adsorption from the inactivated serum than from the active, corresponding to the condition which obtains with chloroform extraction. It has been demonstrated repeatedly, however, that even inactivated sera will produce anaphylatoxins although not so constantly as active sera. The relation of the toxicity of normal guinea pig serum to its antiferment content has already been discussed in a previous paper in which it was shown that the toxicity is proportionate to the degree of removal of the antitrypsin, the serum becoming more toxic with decreasing antitryptic activity. In our present work with bacteria, agar, starch, etc., we have found that the same conditions apply, the agents which absorb most antitrypsin producing the most toxic sera. For these reasons we shall not describe the numerous experiments made in the course of the work.

EFFECT OF TOXINS ON SERUM ANTITRYPSIN.

When we consider the effect produced by true toxins we must, of course, seek a different explanation than one of mere adsorption. Friedberger and Mita (21) demonstrated that an active toxin could be produced by incubating tetanus toxin, diphtheria toxin, and cobra venom with fresh guinea pig complement. Their efforts met

with least success with the cobra venom preparation. They interpreted their results as indicating that the complement had split the toxins to still more toxic products which killed with typical anaphylactic symptoms. In view of the exhaustive work that has been done in the differentiation of the toxins and their components this view as to the unity of toxin action seems at least unreasonable. It has been demonstrated that the toxins have a special affinity for lipoidal substances (de Waele (22), Raubitschek (23)). Whether this depends on the presence of unsaturated fatty acids has not been determined and we are now conducting experiments in this direction. If, then, these lipoidal bonds are first saturated by a toxin we should expect that the trypsin subsequently added would be bound to a lessened degree, since the lipoids are the same in both cases. It is, of course, quite probable that the phenomenon is a purely physical one, but it is interesting to note in this connection the close parallelism shown by the behavior of the toxins and the enzyme in their relation to these lipoids. In protocol I the effect of diphtheria toxin has already been noted.

PROTOCOL II.

No. of tube.	1 per cent. casein solution.	Trypsin.	Serum normal 1 : 10.	Serum 1 : 10 + cobra venom.	Total nitrogen digestion.	Digestion.	
1	2 c.c.	0.0 c.c.			0.0 mg.	0%	
2	2 c.c.	0.2 c.c.			1.65 mg.	100%	
3	2 c.c.	0.2 c.c.	1.0 c.c.		0.28 mg.	17%	Normal.
4	2 c.c.	0.2 c.c.	0.75 c.c.		0.41 mg.	25%	
5	2 c.c.	0.2 c.c.	0.5 c.c.		0.71 mg.	43%	
6	2 c.c.	0.2 c.c.	0.25 c.c.		1.11 mg.	67%	
7	2 c.c.	0.2 c.c.	0.1 c.c.		1.2 mg.	72%	
8	2 c.c.	0.2 c.c.		1.0 c.c.	0.31 mg.	18%	Cobra venom.
9	2 c.c.	0.2 c.c.		0.75 c.c.	0.6 mg.	36%	
10	2 c.c.	0.2 c.c.		0.5 c.c.	0.83 mg.	50%	
11	2 c.c.	0.2 c.c.		0.25 c.c.	1.2 mg.	72%	
12	2 c.c.	0.2 c.c.		0.1 c.c.	1.5 mg.	0.67%	

All tubes were made up to 5 c.c. with normal saline solution.

In our next experiment (protocol II) the results showed that cobra venom[1] also lowered the antitryptic value only to a small extent, a result to be expected from the experiments of Friedberger

[1] The cobra venom was kindly furnished us by Dr. Noguchi.

and Ito, who found it difficult to secure toxic serum in this way, having only one acute death in their series.

The reason why quantitative differences should so markedly influence anaphylatoxin formation is not clear, although we have noted frequently that large amounts of bacteria, kaolin, or starch will adsorb very little of the antitrypsin, while smaller amounts will be effective. That such a condition should be true for bacteria might seem reasonable, for when large numbers are incubated a proportion may die and undergo solution, due to the lipolytic effect of the serum, while the bacterial antiferments, together with the antiferments previously adsorbed from the serum will be thrown back into solution, rendering it more antitryptic than before. That lessened toxicity when larger amounts of bacteria are used should be due to complete protein splitting to atoxic products is out of the question, for even with a concentrated leucoprotease solution Jobling and Strouse (24) found that it required several days to secure highly toxic products from typhoid bacilli.

To show the effects of various amounts of bacteria when incubated with fresh serum, we have carried out the experiment shown in protocol III. The technique was the same as before, with the exception that only one cubic centimeter of serum was used for each mixture, the final dilution being made to ten cubic centimeters, instead of twenty. The number of bacteria used is, of course, not absolute, the measure being a large platinum loop, the amounts used being 4, 2, 1, and ½ loops to each cubic centimeter. From the results it will be seen that the staphylococci are effective only in the larger amounts, while typhoid bacilli give the most marked effects in the one and two loop mixtures, the four loop mixture being less effective.

If serum antitrypsin is adsorbed by the bacteria used in the preparation of anaphylatoxin we should expect that such bacteria would become more resistant to tryptic digestion than untreated bacteria. In order to determine this point the following experiment was carried out.

Typhoid bacilli which had been incubated with fresh guinea pig serum (complement) in the preparation of anaphylatoxin were centrifuged, washed, and dried by grinding in an agate mortar. The dry weight was 30 mg. A similar amount

PROTOCOL III.

No. of tube.	1 per cent. casein solution.	Trypsin.	Serum 1:10 + staphylococci. Loops.				Serum + typhoid. Loops.				Normal serum 1:10.	Total nitrogen digestion.	Diges-tion.	
			4	2	1	½	4	2	1	½				
1	2 c.c.	0.0 c.c.	....	....	....	....	....	....	....	....	....	Trace	0%	
2	2 c.c.	0.2 c.c.	....	....	....	....	....	....	....	....	....	1.54 mg.	100%	
3	2 c.c.	0.2 c.c.	....	....	....	....	....	....	....	....	1.0 c.c.	0.15 mg.	10%	Normal.
4	2 c.c.	0.2 c.c.	....	....	....	....	....	....	....	....	0.5 c.c.	0.41 mg.	26%	
5	2 c.c.	0.2 c.c.	....	....	....	....	....	....	....	....	0.25 c.c.	0.8 mg.	52%	
6	2 c.c.	0.2 c.c.	1.0 c.c.	....	....	....	....	....	....	....	....	0.35 mg.	22%	Staphylococci.
7	2 c.c.	0.2 c.c.	0.5 c.c.	....	....	....	....	....	....	....	....	0.72 mg.	47%	
8	2 c.c.	0.2 c.c.	0.25 c.c.	....	....	....	....	....	....	....	....	1.3 mg.	84%	
9	2 c.c.	0.2 c.c.	....	1.0 c.c.	....	....	....	....	....	....	....	0.26 mg.	16%	
10	2 c.c.	0.2 c.c.	....	0.5 c.c.	....	....	....	....	....	....	....	0.32 mg.	21%	
11	2 c.c.	0.2 c.c.		0.25 c.c.	....	....	....	....	....	....	....	0.85 mg.	55%	
12	2 c.c.	0.2 c.c.	....	....	1.0 c.c.	....	....	....	....	....	....	0.22 mg.	14%	
13	2 c.c.	0.2 c.c.	....	....	0.5 c.c.	....	....	....	....	....	....	0.48 mg.	31%	
14	2 c.c.	0.2 c.c.	....	....	0.25 c.c.	....	....	....	....	....	....	1.4 mg.	90%	
15	2 c.c.	0.2 c.c.	....	....	....	1.0 c.c.	....	....	....	....	....	0.3 mg.	20%	
16	2 c.c.	0.2 c.c.	....	....	....	0.5 c.c.	....	....	....	....	....	0.35 mg.	22%	
17	2 c.c.	0.2 c.c.	....	....	....	0.25 c.c.	....	....	....	....	....	1.4 mg.	90%	
18	2 c.c.	0.2 c.c.	....	....		....	1.0 c.c.	....	....	....	....	0.35 mg.	22%	Typhoid.
19	2 c.c.	0.2 c.c.	....	....		....	0.5 c.c.	....	....	....	....	0.62 mg.	40%	
20	2 c.c.	0.2 c.c.	....			....	0.25 c.c.	....	....	....	....	0.83 mg.	54%	
21	2 c.c.	0.2 c.c.	....			....	....	1.0 c.c.	....	....	....	0.43 mg.	26%	
22	2 c.c.	0.2 c.c.	....			....	....	0.5 c.c.	....	....	....	0.47 mg.	30%	
23	2 c.c.	0.2 c.c.	....			....	....	0.25 c.c.	....	....	....	1.1 mg.	71%	
24	2 c.c.	0.2 c.c.	....			....	....	....	1.0 c.c.	....	....	0.5 mg.	32%	
25	2 c.c.	0.2 c.c.	....			....	....	....	0.5 c.c.	....	....	0.4 mg.	25%	
26	2 c.c.	0.2 c.c.	....			....	....	....	0.25 c.c.	....	....	0.9 mg.	58%	
27	2 c.c.	0.2 c.c.	....			....	....	....	....	1.0 c.c.	....	0.31 mg.	20%	
28	2 c.c.	0.2 c.c.	....			....	....	....	....	0.5 c.c.	....	0.45 mg.	27%	
29	2 c.c.	0.2 c.c.	....			....	....	....	....	0.25 c.c.	....	0.55 mg.	36%	

All tubes were made up to 5 c.c. with normal saline solution.

of dried untreated typhoid bacilli was used as a control. They were each suspended in 3 c.c. of normal salt solution and 10 mg. of sodium carbonate, and 0.1 c.c. of trypsin solution was added to each. The trypsin was of such strength that 0.1 c.c. would digest 2 c.c. of a 1 per cent. casein solution in two hours. The tubes were kept in the incubator over night. The next morning the mixtures were acidified, boiled, filtered through kaolinized hard filters, and nitrogen determinations (Folin) were made to determine the amount of digestion.

No. of tube.	Original typhoid.	Serum-treated typhoid.	Total nitrogen digestion.	Per cent. digestion.
1	3 c.c.	0 c.c.	1.5 mg.	50
2	0 c.c.	3 c.c.	0.375 mg.	12
3	3 c.c.	Total nitrogen = 3 mg.		

The digestion of the untreated bacteria is, therefore, over four times as great as of those bacteria which have adsorbed antitrypsin from the serum.

With staphylococci this difference is not so great, but in the experiments which we have made, the digestion of the untreated bacteria is always somewhat greater than of those which have been treated with fresh serum. We have stated that one can adsorb the antitrypsin from the inactivated serum almost as well as from the active guinea pig serum, although the process seems to be slower. That even inactivated sera will form anaphylatoxins has been shown before (2, 10, 11, 12). From human serum it is much more difficult to remove the antiferment by adsorption. Horse serum antitrypsin we have found almost as easily adsorbable as the guinea pig antitrypsin.

DISCUSSION.

The interpretation of our results is, we believe, simple. The serum proteins are normally prevented from digestion intravitally by the presence of protective lipoids,—unsaturated fatty acids, but when these are removed the proteolytic ferment of the serum can rapidly split the proteins; the exposure of the proteins by partial removal of the antitrypsin through adsorption, or complete removal by means of the lipoidal solvents renders the serum highly toxic to the homologous animal. The recent work of Dold and his coworkers (25) affords a complete confirmation of this view. They state that anaphylatoxins could not be obtained *in vivo* or *in vitro*

with bacteria which had been treated with oils, with bile, or with cholesterol, but that the bacteria had not lost their power to act as antigens, and still contained active endotoxins.

Bacteria treated in this manner have, of course, been saturated with lipoids and can no longer take up more when placed in contact with fresh serum. If the bacteria had been split, as Friedberger contends, then it would be impossible to secure toxic effects *in vivo*, and their antigenic properties would be destroyed. We have repeated the experiments of Dold and his co-workers and have never noted any adsorption of antitrypsin from fresh guinea pig serum by bacteria so treated; neither did the serum so treated become toxic.

The views of Friedberger are, therefore, untenable in that they would make one uniform toxic agent responsible for the manifold toxic effects observed in disease. While split products of proteins (primary proteoses) whether from a foreign source or from the organism's own proteins, are no doubt the causative agents in many instances, the mechanism of their production is unquestionably different from the one supposed by Friedberger. From the complexity of the protein molecule, we should expect that even the higher cleavage products would show differences in their toxic, as they do in their antigenic properties; and the unfailing uniformity of the toxic effects of the anaphylatoxins, together with the lack of antigenic properties, is one of the strongest arguments that the substrate from which the toxic substances are split is the same in every case. That Friedberger (26) himself seems to realize that the position which he has taken is untenable is indicated in his recent work with kaolin, the toxic effects of which he found could be prevented by previous treatment with serum. He says: "The toxicity of kaolin is not due to mechanical injury; it is probably due to the adsorption of certain substances from cells fundamental to the life of the organism."

CONCLUSION.

1. The unsaturated lipoids (serum antitrypsin) can be adsorbed from guinea pig serum, rabbit serum, and horse serum by kaolin, starch, agar, and bacteria.

2. Diphtheria toxin and cobra venom also reduce the serum antitrypsin, possibly because of their affinity for lipoids.

3. Anaphylatoxins represent sera rendered toxic by partial removal of serum antitrypsin.

4. The matrix of the protein split products lies in the serum proteins so exposed.

5. The amount of removal of serum antitrypsin depends on definite quantitative relations; very large amounts and very small amounts of adsorbing substances are least effective (kaolin, starch, and bacteria).

6. Bacteria previously treated with serum or with oils do not adsorb serum antitrypsin.

7. Bacteria treated with serum become more resistant to the action of trypsin.

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COMPLEMENT FIXATION TESTS IN CHRONIC INFECTIVE DEFORMING ARTHRITIS AND ARTHRITIS DEFORMANS.*

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INTRODUCTION.

Recent studies on complement fixation with bacterial antigens have shown that there are distinct possibilities in the method for etiological diagnosis. Koessler (1) and Kolmer (2) applied it in scarlet fever, determining complement fixation with other immune reactions in horses and rabbits immunized with streptococci from scarlet fever patients. In 1904 Besredka (3) applied the test to horses immunized with streptococci. Swift and Thro (4) applied the complement fixation method in attempting to differentiate various forms of streptococci. Smith and Brooks (5) studied this immune body reaction, as well as precipitins, agglutinins, opsonins, and bacteriolysins in rabbits injected with typhoid vaccine, and found that the complement-fixing substance is produced at about the same time and in about the same ratio as other immune bodies. The test has been applied in cholera, glanders in horses, echinococcus invasion, lepra, rabies, and helminthiasis.

The publications of Schwartz and McNeil (6) suggested the possibility of detecting gonococcus infections with secondary joint invasion; and the favorable results with gonococcus vaccine in gonococcus arthritis suggested a possible successful therapy for certain cases. The article by Swift and Thro (4) on the study of streptococci with the complement fixation reaction led us to the employment of this test for bacterial infections other than gonococcal.

If arthritis deformans is an infectious disease, proof of such infection may be searched for as follows:

(1) By making cultures from the exudate in the joints and in tissues about the joints. This method has been followed unsuccessfully by many. The reason is not hard to find if one bears in mind that hematogenous infection of the joint tissues begins in the terminal end bulbs of the blood vessels and in lymph spaces, and

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that it is only when the process invades the joint fluid that bacteriological examinations are positive.

(2) By making cultures from possible foci, evidenced by clinical manifestations, other than the joints.

(3) By making blood cultures for bacteriemia.

(4) By making blood tests for immune bodies against exogenous and autogenous antigens, obtained by (1), (2), or (3). The complement fixation substance (*fixateur* of Besredka) is the most appropriate for this purpose.

It must be borne in mind that (2) and (3) do not locate the infection in or about the joints. Whether or not (3) positively excludes (4) has not been determined, although theoretically this would seem probable. The relation of (4) to acuteness of infection has not been determined, excepting for the gonococcus infection by Schwartz and McNeil. The results in syphilis are not considered, since the Wassermann reaction is not a true complement fixation.

Our results with *Streptococcus viridans* tend to show the findings of Schwartz and McNeil for gonococcus infections; namely, that subacute and chronic processes give positive fixation tests, while the acute processes give negative tests. Some of our control cases of acute sepsis with bacteriemia and subacute and chronic infective endocarditis with bacteriemia gave negative reactions.

Does the complement fixation reaction indicate that the joint lesion is due to the organism giving a positive test? This may not necessarily be so, yet in syphilis the Wassermann test is not positive until the invasion has become systemic, and, as Schwartz and McNeil have shown, positive tests for gonococcus are not obtained until the infection has passed beyond the primary focus. Besredka (3) found that *fixateur* for streptococci of the pyogenic and hemolytic types is readily produced by intravenous injections, and rarely or not at all by subcutaneous inoculations of streptococci in rabbits. That the positive fixation test in infective deforming arthritis indicates that the organism used as antigen is the agent of infection, is borne out (1) by giving small doses of vaccine of the organism, producing thereby focal reactions in and about the joints; (2) by marked improvement when a vaccine of the organism fixed by the patient's serum is given therapeutically over a long period of time;

(3) by finding a primary focus of infection, *i. e.,* infected teeth, prostate, and demonstrating that autogenous antigens, prepared with cultures from an alveolar abscess, yield positive complement fixation tests with the patient's serum and, injected as vaccines in small doses, cause focal reactions.

In other instances cultures of streptococci and staphylococci from the tonsils, endometrium, and sputum have given no reactions with the patient's blood, while positive reactions were obtained with gonococci and *S. viridans* from other sources, and the patients improved under treatment without removal of the tonsils and with the persistence of the endometritis.

BACTERIA ISOLATED AND ANTIGENS USED.

Table I gives the strains of *S. viridans* isolated, their source, the diagnosis, the duration of the disease, and, in the cases of arthritis, the joint involvement. The strains are numbered consecutively as antigens for reference in the records of the complement fixation tests.

Table II gives the antigens and strains of bacteria, other than *S. viridans,* made use of for control complement fixation tests. These have been lettered from A to O.

METHODS EMPLOYED.

Antigens were prepared by suspending a cultural growth in 0.8 per cent. sodium chloride solution. The nature and source of the cultures are shown in tables I and II. Cultures were grown on plain agar, glycerin agar, North's medium (7), and in plain broth. The first generations of *S. viridans* required from two to ten days for growth, and after a few transplantations a suitable growth was obtained within forty-eight hours. The surface growth of from four to eight tubes of solid media was washed off with 0.8 per cent. sterile sodium chloride solution (2 c.c. per tube), shaken thoroughly, and killed by heating to 60° C. for thirty minutes. No advantage was obtained by shaking the suspension from twenty-four to forty-eight hours in an apparatus run by water motor, rather than by thorough shaking by hand. The suspensions were standardized by the method described (1) by Callison (8) by diluting the bacterial suspension with 0.8 per cent. sodium chloride solution, to which had been added carbol-fuchsin, and counting with the Thoma-Zeiss counting chamber, and (2) by Wright's method. The latter method was found to be the most satisfactory, and with an organism as small as *S. viridans* the clearest preparations were obtained by fixing slides with the saturated solution of bichloride of mercury for ten minutes and staining in Loeffler's methylene blue for three minutes.

TABLE I.

Strains of Streptococcus viridans Used as Antigens.

Antigen.	Laboratory No.	Diagnosis.	Source.	Duration.	Involvement.
1	43358	Polyarthritis, chronic	Prostate	15 yrs.; began in left ankle, fingers, and wrists; prostatitis for 15 yrs.	All joints except spine; periostitis; periarthritis; tenosynovitis.
2	46480 46627	Polyarthritis, acute	Alveolar abscess; jaw	9 mos.; began in knees after exposure; rheumatic throat in 1908; abscess of left lower maxilla under last molar in 1912	All joints, cervical spine; periarthritis; tenosynovitis; neuritis; phlebitis, left saphenous and popliteal.
3	48707 48556	Polyarthritis, chronic	Tooth socket, immediately after extraction (2 cultures)	12 yrs.; followed puerperium, 1 mo. after parturition; began in small joints of hands; persistent endometritis	All joints, cervical spine; periarthritis; tenosynovitis; neuritis.
4	50368	Multiple adenitis, chronic	Tooth socket, immediately after extraction, alveolar abscess	14 mos.; adenitis, left cervical, axillary, and mediastinal; excessive enlargement, diagnosed Hodgkin's disease; pyorrhea; anemia; indigestion $4\frac{1}{2}$ yrs.	
5	48970	Polyarthritis, chronic; bronchitis	Sputum	2 yrs.; began in wrists, ankles, and knees	All joints except hips.
6	49347	Tonsillitis, acute	Tonsil	3 dys.; tonsillitis	
7	49384	Tonsillitis, acute	Tonsil	2 wks.; tonsillitis	
8	41979	Infective endocarditis, acute	Blood	5 mos.; acute and chronic endocarditis	
9	52475	Still's disease, 2 yrs.	Tooth socket, immediately after extraction, alveolar abscess	2 yrs.	All joints except spine.
10	51193 51194	Arthritis, monarticular, acute; jaw	Tooth socket, immediately after extraction (2 cultures)	1 yr.; tooth removed after X-ray for apical abscess; later mild polyarthritis	Left temperomaxillary.
11	51292	Arthritis deformans, polyarticular	Tooth socket, immediately after extraction (2 cultures)	30 yrs.; began in fingers, elbows, and knees	All joints; periarthritis; tenosynovitis; neuritis.

TABLE I.—*Continued.*

Antigen.	Laboratory No.	Diagnosis.	Source.	Duration.	Involvement.
12	51189	Septicemia	Blood and spleen, post-mortem		
13	51051	Endocarditis, acute	Blood	2 mos.; endocarditis, acute and chronic	
14	53377	Spondylitis, chronic	Alveolar abscess, pus	9 yrs.; pyorrhea extensive	Spine, thoracic, and lumbar.
15	53466	Erythema, dietetic; pyorrhea	Alveolar abscess, pus	Several yrs.; pyorrhea; dietetic erythema, acute	
16	53465	Pulmonary tuberculosis; pyorrhea	Alveolar abscess, pus	Several yrs.; pyorrhea	
17	52512 (2)	Arthritis, chronic	Tonsil	6 mos.; chronic tonsillitis	Knees.
18	52631	Pyorrhea	Alveolar abscess, extraction	Pyorrhea, several years	
19	52796 (1)	Arthritis, chronic	Alveolar abscess, extraction	20 yrs.; extensive pyorrhea for 12 yrs.	All joints except spine and jaws.
20	52796 (2)	Arthritis, chronic	Alveolar abscess, extraction	20 yrs.; extensive pyorrhea for 12 yrs.	All joints except spine and jaws.
21	52779	Endocarditis, acute infective	Blood	Pyorrhea, several years	
22	53526	Endocarditis, chronic	Alveolar abscess, pus	Chronic endocarditis, duration not known	
23	53524	Endocarditis, chronic	Alveolar abscess, pus	Chronic endocarditis, duration not known; pyorrhea for several yrs.	
24	53668	Arthritis, acute, rheumatic	Alveolar abscess, pus	10 dys.; acute arthritis	Wrists, knees, ankles.
25	54669	Pyorrhea alveolaris	Alveolar abscess, pus	Pyorrhea, several yrs.	
26	51381	Myokymia	Pyorrhea, pus from gums	Pyorrhea, several yrs.; several yrs. recurrent attacks; myokymia for 6 mos.	Small joints and hands.
27	52513	Pyorrhea alveolaris	Alveolar abscess, pus from gums	Pyorrhea, several years	
28	55064	Tonsillitis, chronic	From abscess of tonsil after removal, pus	Chronic tonsillitis for several yrs.; acute abscess of tonsil; tonsillectomy and culture from abscess	

Antigen.	Laboratory No.	Diagnosis.	Source.	Duration.	Involvement.
29	52488	Arthritis and scrofula	Blood	6 mos.; *S. viridans* from blood	All joints ulcerating; probably tuberculosis; scrofula.
30	55240	Arthritis, chronic deforming	Pus from tooth after extraction	3 yrs.; pyorrhea for several yrs. 2 acute exacerbations; all joints involved within a year; *S. viridans* culture from pus from extracted tooth	All joints.
31 and 32	55241 55377	Arthritis, chronic; all joints	Pus from gums Tooth after extraction	9 yrs.; pyorrhea for yrs.; not considered serious by dentist; *S. viridans* from pus from gum and from extracted tooth	All joints.
33	55555	Arthritis, chronic; knees and ankles	Pus from gums	12 yrs.; knees, 12 yrs. and one ankle within last mo.; pyorrhea for years; *S. viridans* from pus from gum and from extracted tooth	Knees and ankle.
34	55854	Arthritis, chronic; knees and ankles	Tooth after extraction		Knees and ankle.
35	51189	Septicemia	Blood	3 wks.; supposed typhoid; *S. viridans* from blood, 3 cultures; cardiac signs normal; recovery under vaccine	

TABLE II.

Streptococci, Excepting Viridans, and Micrococci Used as Antigens.

Antigen.	Laboratory No.	Diagnosis.	Bacteria.	Source.
A			Gonococcus	Polyvalent antigen (Parke, Davis, & Co.).
B	48429	Arthritis deformans	*M. albus*	Cervix uteri.
C	48829	Arthritis deformans	*M. tetragenus*	Cervix uteri.
D	48831	Arthritis deformans	*M. tetragenus*	Cervix uteri.
E	50369	Adenitis, chronic infective	*M. aureus*	Tooth socket.
F	48923	Arthritis, chronic infective	*M. albus*	Sputum.
G	49416	Parkinson's disease	*M. aureus*	Prostate.
H	49765	Arthritis, acute	*S. hemolysans* (*hemolyticus*)	Tonsil.
I			*M. rheumaticus* (Beattie)	Stock.
K		Pneumonia, lobar	Pneumococcus	Stock culture, originally from sputum.
L		Septicemia	*S. hemolysans* (*hemolyticus*)	Blood.
M	53603	Arthritis deformans	*M. albus*	Urine.
N	46377	Endocarditis, chronic infective	*M. zymogenes*	Blood.
O	50251	Ulcerative rhinitis	*M. aureus*	Ulcer of nasal septum.

It was found that for *S. viridans* the best procedure is to dilute the bacterial emulsion to 1 c.c. (1,000,000,000). Of this emulsion from 0.8 to 0.1 c.c. and from 0.09 to 0.01 c.c. were tested for their inhibiting power (anticomplementary) against the standard sheep-rabbit hemolytic unit in 0.1 c.c. quantities with 0.1 c.c. of guinea pig complement corresponding to two units of hemolytic amboceptor and red cells. The complement and different amounts of antigen were incubated in a water bath at 37° C. for forty minutes, and the amboceptor and red cells added; the tubes were again incubated for forty minutes and placed on ice over night. The antigen to be used was selected from the highest limit of dilution of antigen which permitted complete hemolysis. It was found for *S. viridans* that the usable antigen, ten days after preparation, was from 0.03 to 0.01 c.c. (from 10,000,000 to 30,000,000) for the amounts of patients' serum and hemolytic unit used.

It was found that preservatives such as lysol and carbolic acid and alcohol could not be used since these substances interfered with the fixation of the antigen. Considerable difficulty, therefore, was encountered in preparing a permanent antigen. The antigens were kept cold, on ice, when not in use, and with precautions against contamination have often retained their activity for several months. They lose power with age. For a few days after preparation the antigen may be weaker, the usable quantity being as high as 0.09 c.c. (90,000,000), than after standing for a week or ten days, when the usable quantity will be found to be from 0.03 to 0.01 c.c. (30,000,000 to 10,000,000).

For the complement fixation tests 0.03, 0.02, and 0.01 c.c. of patients' serum were used with the amounts of antigen and hemolytic unit previously noted, and

controls were prepared with the same amounts of patients' serum and hemolytic unit and complement, without antigen.

The amount of complement-fixing body (*fixateur*) in patients' serum was found to be small, for often only a partial inhibition of hemolysis occurred with 0.01 c.c. of serum (0.1 c.c. of 1 : 10 dilution), while complete inhibition occurred with 0.02, 0.03, 0.04, and 0.05 c.c. of a positive serum which was weak in anti-complementary power.

The necessity for the employment of so many streptococcus antigens is due to the facts that a group reaction for several strains could not be obtained; without preservation it is difficult to prevent contamination, and the retention of fixability is variable. As a rule antigens freshly prepared and two or three weeks old give good fixation, within narrow limits.

It has not always been possible to carry through a known positive serum, but several sera have been tested at a time and a known negative serum used as a control, the negative or positive nature of the control sera having been tested in comparison with rabbit sera, recorded in the report on animal experiments (9).

COMPLEMENT FIXATION TESTS IN ARTHRITIS. RESULTS POSITIVE FOR STREPTOCOCCUS VIRIDANS. SEVENTEEN CASES.

In table III are recorded the results of positive complement fixation tests for *S. viridans* with sera from patients afflicted with chronic infective deforming arthritis. The qualification "chronic infective deforming" is applied because the positive reactions indicate the infective nature of the disease. Cases 1, 8, 9, 10, and 16 began as mildly febrile infections in one or more joints, rapidly extended within a few weeks to most of the large and small joints, and ultimately produced the joint deformities—and the joint lesions shown by radiographs in cases 1 and 10,—found in classical cases of arthritis deformans.

Cases 8 and 9, with hepatomegaly and splenomegaly, in females of nine years, were typical of Still's Disease.

Cases 7, 10, 13, and 14 only were males; the remaining thirteen were females. Ages ranged from 30 (case 15) to 69 years (case 16). Duration, at first visit, was from 9 months (case 1) to 30 years (case 5). In fifteen cases, all except cases 7 and 13, foci of infection were found. Cultures of *S. viridans* were isolated from eleven cases, in ten from the teeth, and in one from the sputum. In one case (No. 3), *Micrococcus tetragenus* was isolated from uterine curettings, and in five cases no cultures were taken. In four cases with no cultures there was a history of pyorrhea alveolaris in three and a history of acute rheumatism in one.

TABLE III.

Sera Reacting to Streptococcus viridans.

Cases of arthritis deformans and Still's disease.	Age in yrs.	Date.	Wassermann reaction.	Gonococcus infection.	Cases of infective deforming arthritis: bacterial antigens. 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	A	B	C	D	E	F	G	H	I	K	N	Culture from foci.
1. Mrs. C.	38	vi–11	0	0	o	+																														*S. viridans* + from abscess of jaw.
							Autogenous																													
2. Mrs. L.	41	v–13	0	0	o	o			o			o			o	o		+	o						o	o	o						o	o		*S. viridans* + from tooth socket and sacculi.
		i–3–12	0	0		+	+ o	o																												
							Autogenous																													
3. Mrs. B.	43	i–15–12	0	0	o	+	+																			o										*M. tetragenus* from cervix and curettings.
		ii–12	0	+		+ o	+	o			+	o	+	o											+	o			+	o						
4. Mrs. S.	40	iv–12	0 +	0	o	o	o		+	o	o														o	o		o	o	o						*S. viridans* + from pus from gums.
5. Miss L.	60	v–12	0	0	o+	+			o	o	o	o													o	o		o	o	o	+ o					*S. viridans* from tooth extracted.
6. Mrs. W.	40	x–12	0	0	o	o	+		o		+	o													o	o		o	o	o	+	o	o			*S. viridans* + from tooth pulp, socket, sacculi from gum.
		iv–13	0	0	o	o			o			o		+		o																				
														Autogenous																						
7. F. S.	41	iii–12	0	+	+																				o											None.
8. O'D.	9	ii–12	0	0		+			+					+											o								o			*S. viridans* from pus from gums. Still's disease.
9. S.	9		0	0									+																							*S. viridans* from pus from gums. Still's disease.
													Autogenous																							
10.[1] P.	49	ii–12	0	+		o	+ o	+ o	+																+	o			+ o	o						*S. viridans*, *B. influenzæ*, *M. aureus* in sputum.
									Autogenous																											
11. R.	48	vi–13	0	0			o	o	o	+	o	o	o	o	o										o											Alveolar abscess, cured; no cultures.
12. M.	50	iii–13	0	0	o	+						o													o			o	o	+	o		o			Pyorrhea alveolaris, cured; no cultures.
13. S.	?	v–13	0	0	o	o			o			o			o	o									o	o		o					+	o		Acute rheumatism years ago; no cultures.
14. C.	46	x–13	0	0												o				+				+	o								+		o	Pyorrhea alveolaris, cured; no cultures.
15. G.	30	x–11	0	0																					o											Pyorrhea alveolaris, cultures from teeth and *M. albus* from cervix uteri.
		xi–11	0	0																					o											
		xi–13	0	0												o				+				+	o								o		o	

Cases of arthritis deformans and Still's disease.	Age in yrs.	Date.	Wassermann reaction.	Gonococcus infection.	Cases of infective deforming arthritis: bacterial antigens.														Culture from foci.
					1	18	20	25	28	29	30	31	32	33	34	36	I	N	
16. Mrs. B.	69	xi–13	0	0	o	...	...	+	..	+	..	..	..	..	..	..	o	o	Pyorrhea alveolaris, extensive; *S. viridans* from pus from gums and from tooth sockets.
17. Mrs. McM.	60	ii–14	0	0	o	o	o	...	+	o	o	o	+	+	o	+	o	o	Pyorrhea alveolaris, extensive; *S. viridans* from pus from gums and from tooth sockets.

[1] Case 10 gave also a positive reaction for gonococcus infection (table IV, case 20), and improved under gonococcus vaccine. The positive fixation test to the autogenous antigen (No. 5) of *S. viridans* from the sputum was probably due to the chronic infection in the bronchi.

In two cases not recorded since the blood was not tested for complement fixation, *S. viridans* was isolated from the prostate.

In five cases (1, 2, 6, 9, and 10) tests against autogenous antigens of *S. viridans* were positive. Two cases (13 and 14) gave positive reactions against *M. rheumaticus*. Cases 3, 7, and 10 gave positive tests for gonococcus infection and thus caused confusion in respect to a choice of vaccine for specific therapy. Cases 3 and 7 did not improve under injections of gonococcus vaccine and improved under *S. viridans* vaccine; case 10 improved under gonococcus vaccine, and the positive reaction to autogenous *S. viridans* might be explained by the infection in the bronchi, since *S. viridans* was isolated from the sputum. Treatment of cases 7 and 10 resulted in what should be termed a cure, with deformity but with unusual restoration of function. Cases 3, 5, 6, and 10 gave positive tests for *M. aureus,* and case 12 for *M. albus;* in case 10 only, with a positive culture of *M. aureus* from the sputum, could a probable focus of infection for *M. aureus* be found. What relation these micrococci might have had to the arthritis was not determined.

ABSTRACTS OF CASES OF ARTHRITIS DEFORMANS GIVING POSITIVE FIXATION TESTS FOR STREPTOCOCCUS VIRIDANS.

Case 1.—Mrs. C., aged 38 years. Duration, at first visit, June, 1911, 9 months. Began in knees after exposure to wet and cold (rheumatic throat) in 1908; abscess of left lower maxilla under last molar tooth. Feb., 1912. Clinical course of infective disease with pyrexia for seven months; all joints involved including cervical spine and both temperomaxillary joints; periarthritis; tenosynovitis; neuritis; phlebitis of left saphenous and popliteal. Oct., 1913. condition typical of arthritis deformans.

Case 2.—Mrs. L., aged 41 years. Duration, at first visit, Jan. 1, 1912, 12 years. Began in small joints of hands one month after parturition with pyrexia; persistent endometritis; all joints including cervical spine; periarthritis; tenosynovitis; neuritis. *S. viridans* from tooth extracted in 1912.

Case 3.—Mrs. B., aged 43 years. Duration, at first visit, Feb., 1912, 4 years. Began insidiously in knees, ankles, and wrists; persistent endometritis; all joints including cervical spine and left temperomaxillary joint; periarthritis; tenosynovitis; neuritis; phlebitis of left saphenous vein; pleuritis.

Case 4.—Mrs. S., aged 40 years. Duration, at first visit, Apr., 1912, 4 years. Began insidiously in knees and wrists; pyorrhea alveolaris for years; all joints involved, except spine and jaws.

Case 5.—Miss L., aged 60 years. Duration, at first visit, May, 1912, 30 years. Began insidiously in fingers, elbows, and knees; pyorrhea alveolaris for years,

whether or not preceding arthritis is not known; all joints including cervical spine and temperomaxillary joints; periarthritis; tenosynovitis; neuritis; extensive crippling and marked deformity. Thrombophlebitis and gangrene of one leg and toes. Death, Feb., 1914.

Case 6.—Mrs. W., aged 40 years. Duration, at first visit, Oct., 1912, 1 year. Began in left temperomaxillary joint; radiograph showed apical abscess over left upper molar; mild polyarthritis.

Case 7.—F. S., male, aged 41(?) years. Duration, at first visit, Mar., 1912, 1½ years. Began in ankle and wrists; one elbow, small bones of right hand, and small joints of feet involved; chronic prostatitis; gonococcus vaccine for three months before first visit; no improvement; *S. viridans* vaccine; marked improvement and cessation.

Case 8.—O'D., female, aged 9 years. Duration, at first visit, Feb., 1913, 3 years. Still's disease; all joints involved, except spine; deformity typical of arthritis deformans.

Case 9.—S., female, aged 9 years. Duration, at first visit, Nov., 1912, 2 years. Still's disease; all joints involved, except spine; deformity typical of arthritis deformans.

Case 10.—P., male, aged 49 years. Duration, at first visit, Feb., 1912, 2 years. Previously tonsils and teeth inspected and teeth treated; tonsils not treated. Began in wrists, ankles, and knees; all joints involved except hips and temperomaxillary joints; complement fixation tests positive to gonococcus and *S. viridans;* deformity more typical of gonococcus arthritis than of arthritis deformans; recovery under treatment with gonococcus vaccine.

Case 11.—Mrs. R., aged 48 years. Duration, at first visit, June, 1913, 12 years. Began in toes, extended to all joints of left foot and then right foot; could not walk for a year; rheumatism 18½ years before first visit, during pregnancy and puerperium; all joints involved except the spine; left internal ear painful,—diagnosed "rheumatic ear"; radiograph showed apical abscess of right upper molar, which was treated and pronounced cured one month before first visit; deformity moderate.

Case 12.—Miss M., aged 50 years. Duration, at first visit, Feb., 1913, 5 years. Began in left knee; shortly followed to right knee and all joints, excepting left jaw and hips; progress slow with exacerbations of pain, redness, increased swelling, and local heat in joints; deformity moderate.

Case 13.—S., male, age not known. Duration, at first visit, May, 1913, several years, following acute rheumatic arthritis years before; all joints involved; deformity typical of arthritis deformans.

Case 14.—T. C. C., male, aged 46 years. Duration, at first visit, Oct., 1913, 8 years. Began in knees; all joints except right shoulder involved; deformity and contractures marked; perisynovitis; neuritis and extreme emaciation.

Case 15.—Mrs. R. G., aged 30 years. Duration, at first visit, Nov., 1911, 8 years. Began in knees and wrists; all joints, except spine and hips, involved; pyorrhea alveolaris and endometritis for years; arthritis slowly progressive with exacerbations.

Case 16.—Mrs. B., aged 69 years. Duration, at first visit, Nov. 29, 1913, 6 years. Acute rheumatism at 6 years of age; chronic arthritis began in 1908;

an acute attack with low fever for a few days and inflammation of left thumb, followed by small joints of both hands, wrists, shoulders, hips, knees, maxillary joints, cervical spine, and ankles; course progressive with exacerbations; neuritis in left arm two years ago; teeth considered excellent until two years ago; considered good at present, although pyorrhea is extensive, involving six or more teeth; indigestion and foul stools for years. Died of Bright's disease and uremia, Mar., 1914.

Case 17.—Mrs. McM., aged 60 years. Duration, at first visit, Feb. 12, 1914, several years. Began in ankles and knees; involved subsequently small joints of hands and feet, spine, shoulders, elbows; pyorrhea alveolaris extensive for several years.

In the same case one may find hypertrophic, atrophic, periarthritic, and often tenosynovitic lesions; and multiplicity of lesions is noticeable in the acute and chronic infective cases which end ultimately in the progressive deforming arthritis without evidences of infection. In two cases only, not recorded in table III, since the progress of the disease and the deformity were not of arthritis deformans, have the symptoms and signs been those of an atrophic arthritis confined to the joint surfaces, with creaking and crepitation on motion, little tenderness, no pain, no local temperature, and with ultimate erosion of the small joints; one was a female, aged 44 years, and one a male, aged 40 years, and in both the progress was slow, over a period of three or four years with no annoyance other than that from the creaking and grating and the partial loss of control of the small joints.

RESULTS POSITIVE FOR GONOCOCCUS.

Table IV gives the results with eight cases reacting to gonococcus antigen. It may be assumed that the tests in cases 18 and 19 were incomplete since no *S. viridans* antigens were used. Four cases (18, 19, 20, and 21) exhibited deformity characteristic of arthritis deformans. Two of these four cases (Nos. 2 and 21) were classed as infective deforming arthritis and are included under the cases in table III, since both reacted also to *S. viridans*.

Table V records three cases of multiple arthritis, giving positive Wassermann reactions and recovering under antisyphilitic treatment. The positive gonococcus reaction in case 26 (table IV, case 24) indicated no causative relation to the disease.

TABLE IV.

Sera Reacting to Gonococcus Antigen.

Cases of arthritis deformans.	Wassermann reaction.	Gonococcus.	Bacterial antigens. 1	2	3	4	5	6	7	8	9	10	11	A	B	E	F	Culture from foci.
18. J. P.	o	+												+				None.
19. K.	o	+												+				None.
20. P.[2] (10)	o	+	o	o	+ o	+ o	(+ Autogenous)							+	o	+ o	o	*S. viridans* from sputum. Staphylococcus and *B. influenzæ* from sputum.
21. Mrs. B.[3] (3)	o	+		+ o	+	o			+	o	+	o		+	o	+	o	*M. tetragenus* from cervix uteri.
Infective deforming arthritis.																		
22. H.	o	+			o									+		o		*M. albus* from bladder.
23. C.	o	+		o					o	o				+				None.
24. D.[4] (26)	+	+	Luetic arthritis											+	o			None.
25. P.	o	+	o		o	o		o	o	o	o	o	o	+				None.

TABLE V.

Sera Reacting for Lues.

Polyarthritis, periostitis, luetic.	Wassermann reaction.	Gonococcus.	Bacterial antigens. 1	2	3	4	5	6	7	8	D	E	F	H	Culture from foci.
26. D.[5]	+	+							o	o					None.
24.															None.
27. E.	+	o	o	o	o		o		o	o		o			None.
28. W.	+	o	o	o	o		o	o	o	o	o	o	o	o	None.

COMPLEMENT FIXATION TESTS IN ARTHRITIS. RESULTS NEGATIVE.

Table VI records the tests in eighteen cases of arthritis which should be classed as arthritis deformans. In eight cases (Nos. 29,

[2] Case 20 gave a positive reaction for *S. viridans* and is recorded in table III also (case 3).

[3] Case 21 gave a positive reaction for *S. viridans* and is recorded in table III also (case 10).

[4] Case 24 gave a positive Wassermann reaction and is recorded in table V also; recovered under antisyphilitic therapy.

[5] D. gave a positive reaction for gonococcus infection and is recorded also in table IV. A fourth case (A. L., female) giving a positive Wassermann reaction is not recorded since the arthritis was monarticular (Charcot's joint).

30, 31, 32, 33, 34, 35, and 36) tests against *S. viridans* were not made, and it might be assumed that some of these would give positive results.

Summary.—Of 43 cases[6] of deforming arthritis, 25 (58 per cent.) gave positive evidence by complement fixation and Wassermann tests of their infective nature; 19[7] cases (44 per cent.) of arthritis deformans (including 2 cases of Still's disease) gave positive tests, and of these 19 cases, 17 (39 per cent.) reacted positively to *S. viridans* antigens, and 4 (9.3 per cent.) positively to a polyvalent gonococcus antigen.

TABLE VI.

Arthritis Deformans. Sera Not Reacting to Complement Fixation Tests.

Case No.	Cases of arthritis deformans.	Wassermann reaction.	Gonococcus infection.	Bacterial antigens.																				
				1	2	3	4	5	6	7	8	9	10	11	12	B	D	E	F	G	H	I	K	O
29	H. McN.	0	0																					
30	B. McG.	0	0(2)																					
31	M. B.	0	0(2)																					
32	A. H.	0	0																					
33	J. T.	0	0																					
34	A.K. ix–10	0	0																					
	vi–11	0	0																					
35	E. G.	0	0(2)		0							*M. tetragenus* from cervix uteri.												
36	R.	0	0																					
37	H.	0	0	0	0		0			0	0		0											
38	McM.	0	0	0	0			0			0			0	0							0		
39	Mrs. M.[8]	0	0	0	0	0		0	0	0	0					0	0	0	0	0				0[9]
40	P. ix–12	0	0	0	0	0		0		0	0		0		0		0	0	0	0	0			
	x–12	0	0	0	0	0		0					0				0	0	0					

Case No.	Cases of arthritis deformans.	Wassermann reaction.	Gonococcus infection.	Bacterial antigens.																		
				1	2	5	8	11	12	14	15	16	17	18	19	20	21	22	A	B	I	K
41	S.	0	0	0	0	0	0	0	0	0[9]	0										0	0
42	B.	0	0	0	0	0	0	0	0			0							0	0		
43	P.	0	0	0	0	0		0	0	0	0	0	0	0								0
44	H.	0	0	0	0	0		0	0	0	0	0	0	0								0
45	D.	0	0									0			0	0	0	0				
46	S.	0	0						0			0			0	0	0[9]	0			0	

[6] Total in tables, 46 cases; as 3 cases are counted twice, the total number of cases is 43.

[7] Total in tables, 21 cases; as 2 cases are counted twice, the total number of cases is 19.

[8] Negative to autogenous antigen (*S. aureus*) from ulcer of nasal septum.

[9] Autogenous.

Three cases of multiple arthritis and periostitis of the small joints of the hands and the distal ends of the long bones of the forearms and legs reacted to the Wassermann test and recovered under antisyphilitic therapy.

Summary.—Of 37 cases of arthritis deformans, recorded in table IV, exclusive of case 10, and three cases of luetic arthritis and four cases of infective deforming arthritis due to gonococcus, 19 (51 per cent.) gave positive reactions for complement fixation; of these 19 cases, 16 (43 per cent.) with *S. viridans*, and 3 (8.1 per cent.) with gonococcus antigens.

Blood Cultures.—Cultures of blood withdrawn from the median vein were negative in every case, but little importance can be attached to the negative results, since in two cases only was more than one culture taken.

SUMMARY OF CASES.

Positive.	Complement fixation.	
Arthritis deformans	Cultures from foci.	Autogenous antigen.
S. viridans[10]	15 cases	Positive in 5 cases.
16 cases	2 cases	
Gonococcus[11]	(Not gonococcus)	
3 cases		
Chronic infective deforming arthritis		
Gonococcus[12]		
4 cases		
Wassermann		
3 cases		
Total 26		
Negative.		
Arthritis deformans		
18 cases		
Total cases 44		

COMPLEMENT FIXATION ON CONTROL CASES.

NOT ARTHRITIS.

Table VII records complement fixation tests for strains of *S. viridans* and for a polyvalent gonococcus antigen and Wassermann

[10] Case 70 proved to be a gonococcus infection and is taken from the 17 cases in table III and placed in table IV (case 22).

[11] Case 21 proved to be a *S. viridans* infection and is taken from the five cases in table IV and placed in table III (case 3).

[12] Case 24 proved to be a case of luetic arthritis and is taken from the four cases in table IV and placed in table V (case 26).

TABLE VII.

Controls.

Case No.	Diagnosis.	Wassermann reaction.	Gonococcus.	Bacterial antigens. 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	21	23	25	28	29	30	31	A	B	C	E	F	G	I	K	N	Remarks.
47	Acute rheumatic arthritis	0	0	o	o						o			o																				o		+	o		
48	Acute rheumatic arthritis	0	0	o																		o			o	o	o	o	o							o			
49	Acute rheumatic arthritis	0	0	o																		o			o	o	o	o	o							o			
50	Acute rheumatic arthritis	0	0	o	o							o				o					o	o			o					o	o	o							*S. viridans* from pus and from gums; autogenous antigen 24, anticomplementary.
51	Acute infective endocarditis, malignant	0	0	o	o		o																								o		o	o					*S. viridans* from blood.
52	Acute infective endocarditis	0	0	o	o		o																								o		o	o					*S. viridans* from blood.
53	Chronic infective endocarditis	0	0	o	o		o																																*S. viridans* from blood.
54	Septicemia	0	+	o	o																																		*S. hemolyticus* from blood.
55	Chronic endocarditis, aortic	+	0	o	o								o	o		o																				o	o		
56	Chronic endocarditis, v-12-13, mitral	0	0	+	o							o				o					o	o	o	o						o	o	o							*S. viridans* from pus from gums; autogenous antigen 22, anticomplementary.
	v-19-13, mitral	0	0	+	o							o				o					o	o	o	o						o	o	o							
57	Chronic endocarditis, (with nephritis) v-12-13	0	0	o	o							o				o					o	o	o	o						o	o	o							*S. viridans* from pus from gums; autogenous antigen 23, negative.
																							Autogenous															
	v-19-13	0	0	o	o							o				o					o	o	o	o						o	o	o							
58	Chronic endocarditis	0	0	o	o			o			o			o	o																					o			

Case No.	Diagnosis.	Wassermann reaction.	Gonococcus.	Bacterial antigens. 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	21	23	25	28	29	30	31	A	B	C	E	F	G	I	K	N	Remarks.
59	Acute tonsillitis.......	o	+	o	o	o				o																													*S. viridans* from tonsil; antigen 7; prostatitis.
60	Chronic infective adenitis (ii-18-12), simulating Hodgkin's disease (vi-20-13)	o	o	–	o	+ o	o	o																							o		o	o					*S. viridans* from pus from gums and from extracted teeth.
		o	o	+	o	+	–	+	o	o	o	o																					o	o					
61	Pyorrhea alveolaris, influenza............	o	o	o	o			o			o			o	o																					o			
62	Pyorrhea alveolaris, lobar pneumonia....	o	o	o	o			o			o			o	o	o															o	o				o	o		
63	Pyorrhea alveolaris, pulmonary tuberculosis	o	o	o	o			o			o			o	o		o	o																		o	o		*S. viridans* from pus from gums.
64	Pyorrhea alveolaris, pulmonary tuberculosis.	o	o	o	o			o			o			o	o																					o	+		
65	Pyorrhea alveolaris, erythema.............	o	o	o	o			o			o			o	o		o	o																		o	o		*S. viridans* from pus from gums; autogenous antigen 14, negative.
																	Autogenous																						
66	Anemia, secondary....	o	+	o	o	o		o	o																								o	o	o				
67	Myeloma, Bence-Jones proteinuria.........	o	o	o	o			o						o	o		o	o	o	o	o																o		Osteoporosis of spine; Bence-Jones proteinuria.
68	Sciatica..............	o	o	o	o						o			o																				o		o	o		
69	Lumbago............	o	o	o	o	o		o	o	o	o																						o		o				
70	Lumbago............	o	o																			o			o		o									o		o	Lumbago (spondylitis?), neurasthenia.
71	Paralysis agitans......	o	+	o	o	o																																	*M. albus* from prostate.
72	Paralysis agitans......	o	o																						o	o	o	o	o										
73	Myasthenia gravis.....	o	o																			o			o		o									o		o	

tests in a miscellaneous group of cases not afflicted with arthritis. One (case 47) of four cases (Nos. 47, 48, 49, and 50) gave a positive reaction against *M. rheumaticus.* Two cases (Nos. 50 and 51) of acute infective endocarditis gave negative tests at the time that the *S. viridans* was isolated from the blood. Case 54, of septicemia, gave a positive fixation test for gonococcus, and *Streptococcus hemolyticus,* the causative agent, was isolated from the blood. Three other cases (Nos. 59, 66, and 71), one of acute tonsillitis and chronic prostatitis, one of secondary anemia, and one of paralysis agitans, in males, gave positive reactions against gonococcus, and as with case 54 the positive blood reactions bore no relation to the affliction complained of and should be considered as positive evidence of clinically latent gonococcus infection which one might suppose to be relatively common on account of the high incidence of gonococcus infection, particularly in the male. In this case of acute tonsillitis (No. 59) *S. viridans* was isolated from the surface of the tonsil, and the complement fixation test for *S. viridans* was negative.

One case (No. 55) of chronic endocarditis involving the aortic valve gave a positive Wassermann reaction, and one (No. 56) involving the mitral valve gave twice a positive reaction to *S. viridans* (antigen 1).

In one case of multiple adenitis, simulating Hodgkin's disease, the blood reacted positively to three strains (antigens 1, 3, and 5) of *S. viridans,* but not to autogenous antigen (No. 4) of *S. viridans* from pus from the gums and from the sacculi on extracted teeth.

One case (No. 64) of pulmonary tuberculosis with secondary infection, and with extensive pyorrhea alveolaris reacted to the pneumococcus, possibly in response to the secondary infection in the lung, which can be surmised only since there is no record of a culture from the sputum.

Cases 61, 62, 63, 64, and 65, of extensive pyorrhea alveolaris and suffering from and under observation for diseases undoubtedly not due to a type of streptococcus, were negative to tests for *S. viridans.*

On the whole, it should be noted that the 26 control cases were consistently negative to complement fixation tests for *S. viridans* and that the three cases (Nos. 47, 56, and 60) which reacted to

M. rheumaticus and *S. viridans,* two organisms closely related if not strains of one organism as will be shown in another paper by Dr. Thro, were probably infected with strains belonging to the *S. viridans* group.

CONCLUSIONS.

1. From a comparison of the results with arthritis deformans recorded in tables III, IV, and VI, and with the control cases recorded in table VII, one is justified in concluding that *Streptococcus viridans* is an infectious agent and excites the production of a complement-fixing substance (*fixateur*) in the organism in cases of arthritis deformans, and, therefore, *Streptococcus viridans* is the probable causative agent of the disease in many cases of arthritis deformans. Probably 40 per cent. and more of cases of arthritis deformans should be considered as chronic infective deforming arthritis.

2. Rarely the clinical manifestations of arthritis deformans may be due to gonococcus infection.

3. The serum from one case may react positively to two different organisms, most frequently to *Streptococcus viridans* and to gonococcus, when the reaction to the former should be considered the indicator of the causative agent, since with the latter infection is often latent in the genito-urinary tract. Incidence of gonococcus infection is high and the clinical manifestations of arthritis deformans are rarely produced by gonococcus infection.

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CONCERNING A POLYVALENT ANTIGEN FOR THE COMPLEMENT FIXATION TEST FOR STREPTOCOCCUS VIRIDANS INFECTION.*

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The following experiments were conducted to determine the time of appearance of complement fixation substances in rabbits. In making control tests with normal rabbits before streptococcus vaccine was injected, it was found that rabbits may have in small amount a natural *fixateur* to *Streptococcus viridans,* since some tests were positive. Normal rabbits did not react to staphylococci. Complement fixation became positive in eleven days. The disturbance due to the anticomplementary action of some rabbit serum is well known.

Mar. 8, 1913, 11 A. M. Seven mice were inoculated as shown in table I. Antigen 2 was prepared from a few days' growth of *S. viridans,* isolated from an abscess of the left lower jaw in a case of acute infective deforming arthritis, later typical arthritis deformans. The growth was suspended in 0.8 per cent. saline solution and killed by heating to 60° C. for thirty minutes and standardized by Wright's method and diluted so that 1 c.c. contained 100,000,000 streptococci. Antigen 11 was isolated from an alveolar abscess in a case of arthritis deformans and was prepared as antigen 2. Antigens 2 and 11 had been used for the vaccinations of cases of arthritis deformans and had given positive complement fixation tests with blood from patients suffering with arthritis deformans.

TABLE I.

Mouse No. Mar. 8. 1913.	Antigens killed by heating.				Result.
	S. viridans 2.	*S. viridans* 11.	*M. rheumaticus* (Beattie).	Pneumococcus.	
275	10,000,000				No effect.
259	50,000,000				No effect.
251		10,000,000			No effect.
276		50,000,000			No effect.
236			50,000,000		No effect.
260				10,000,000	No effect.
252				50,000,000	No effect.

* Received for publication, April 20, 1914.

The *M. rheumaticus* antigen was a strain obtained from the New York Board of Health Laboratories and originally came from the laboratory of Dr. J. M. Beattie, of Edinburgh. The pneumococcus antigen was prepared from a moderately virulent strain obtained from the blood of a case of lobar pneumonia. The *M. rheumaticus* and pneumococcus antigens were prepared and standardized and diluted as antigens 2 and 11.

Mar. 25, 1913. The same seven mice were inoculated as shown in table II. Antigen 10 was prepared from a culture from the tonsil of a case of infective arthritis. All the antigens were ten times the strength of those inoculated on Mar. 8, and five were killed by heating to 60° C. for thirty minutes. Antigen 13 and pneumococcus were isolated from the blood, grown on North's medium and not killed. The pneumococcus antigen was from a twenty-four hour growth, and was the same as the pneumococcus antigen killed by heating to 60° C.

TABLE II.

Mar. 25, 1913.	Antigens killed by heating.					Antigens not killed.		Result.
	S. viridans 2.	*S. viridans* 10.	*S. viridans* 11.	*M. rheumaticus.*	Pneumococcus 10.	*S. viridans* 13.	Pneumococcus 10.	
75	500,000,000	....	....	....	....	....	....	No effect.
59	....	500,000,000	....	....	....	....	....	No effect.
51	....	....	500,000,000	....	....	....	....	No effect.
76	....	....	....	500,000,000	....	....	....	No effect.
36	....	....	....	....	500,000,000	....	....	No effect.
60	....	....	....	....	....	500,000,000	....	No effect.
52	....	....	....	....	....	....	500,000,000	Death after 72 hrs.

The mouse inoculated with the living twenty-four hour culture of pneumococcus died after seventy-two hours. The other inoculations in doses of 500,000,000 were without effect, even the inoculation of 500,000,000 living *S. viridans.*

On Mar. 28, three days after the injection of 500,000,000, two of the mice, Nos. 259 and 251, were inoculated with 0.75 c.c. of living polyvalent antigen prepared from fourteen strains of *S. viridans* of five days' growth. The dosage in millions by Wright's method was not determined, but was several times stronger than that of Mar. 25, since the cultures were grown on 2 per cent. glycerin agar tubes from 2 to 3 cm. in diameter, and the surfaces of these fourteen large tubes were washed with 20 c.c. of saline solution. For the 500,000,000 dilution the surface of a small agar tube was washed with 2 c.c. of saline solution.

It is safe, therefore, to assume that the stronger antigen was ten times 500,000,000, or 5,000,000,000. The possibility of immunity in the mice from previous injections, on March 8 of 50,000,000, and on March 25 of 500,000,000, was wanting, since the 500,000,000 dose which might have caused such immunity was given three days before the dose of 5,000,000,000. From the dose of 50,000,000 on March 8, after an interval of twenty days one would expect

anaphylaxis rather than immunity. Subsequently these mice were injected again with 5,000,000,000 living *S. viridans* without any apparent effect.

The virulence of living *S. viridans* having proved impotent for mice, rabbits were inoculated intravenously with large doses of living antigen, prepared from fourteen strains grown in large tubes of 2 per cent. glycerin agar for four days. On May 28 four rabbits were inoculated, as shown in table III, three with one third of the antigen, and a fourth with the saline solution suspension of the growth of *M. albus* from one agar tube.

TABLE III.

Rabbit No. May 28, 1913.	Polyvalent antigen not killed, 14 strains, *S. viridans*.	*M. albus* antigen not killed, 1 agar culture in 1 c.c.
521 intravenous	5.0 c.c.	
482 intravenous	5.0 c.c.	
244 intravenous	5.0 c.c.	
498 intravenous		1.0 c.c.
Repeated June 5	7.0 c.c.	1.0 c.c.
Repeated June 11	8.0 c.c.	2.5 c.c.
Repeated June 19	8.0 c.c.	3.0 c.c.

On June 3, six days after the first inoculation, blood was examined and the rabbit serum tested against eleven of the fourteen strains for immunization, for complement fixation substance, with the results shown in table IV.

For the complement fixation tests the serum was diluted 1 : 10, and 0.3, 0.2, and 0.1 c.c. were used in three tubes, indicated by the three rows of + or 0 signs.

The antigens were killed by heating to 60° C. for half an hour, standardized in the usual manner in the forenoon of the day on which they were used, and the strength in millions was determined by Wright's method. It was found that as a rule an antigen of small strength test (1 c.c. = 1,000,000,000) should be diluted 1 : 10, and from 0.3 to 0.1 c.c. used against from 0.3 to 0.1 c.c. of serum.

From table IV it is seen that six days after a single inoculation of large doses of living *S. viridans* polyvalent antigen, the complement fixation substance is present in rabbit serum in small amount, and that the reactions are variable and incomplete against each of the strains used for immunization.

On June 5, 11, and 19, the same four rabbits were inoculated in the same manner; three with the living antigen prepared from fourteen strains of *S. viridans* and one with *M. albus* antigen.

TABLE IV.

Rabbit No.	Wasser-mann reaction.	Gono-coccus.	*S. viridans* antigens.										
			1	2	3	4	5	6	7	8	9	10	11
521	+	+	Inhibition in control	Inhibition in control	o	+ o o + + o + + +	o	o	+ o o + o o + o o	+ o o + + o + + +	o o o + o o + + o	+ o o + o o + + +	+ + o + + + + + +
482	+	+	Inhibition in control	Inhibition in control	+ + o + + + + + +	+ + o + + + + + +	+ + o + + + + + +	o o o + o o + + +	o	+ + o + + + + + +	o o o + + o + + +	+ + o + + + + + +	+
244	+	+	Inhibition in control	Inhibition in control	+ o o + o o + + o	+ o o + + o + + +	+ + o + + o + + o	o o o + o o + o o	+ o o + + + + + +	+ o o + o o + + +	+ + o + + + + + +	o o o o o o + + o	+ + o + + + + + +
498 *M. albus*	+	+	+	+	+	+	+	+	+ + o + + + + + +	+	+	+	+

+ indicates slight hemolysis; + +, marked hemolysis; + + +, complete hemolysis; o indicates inhibition. A single large + or o indicates complete hemolysis or inhibition in three tubes.

On June 18, seven days after the third inoculation, the rabbit serum was tested with the results shown in table V.

TABLE V.

Rabbit No.	Wassermann reaction.	Gonococcus.	Compound antigen.		Antigen 5.
			1	2	
521	+	+	+	+	+
482	+	+	+	ooo + oo + + o	+ oo + + + + + +
244	+	+	+	+	+
498	+	+	+	+	ooo + oo + + +

The rabbit serum was found to inhibit, as shown in table VI, and this anticomplementary action was neutralized by using two units (0.2 c.c.) of complement.

TABLE VI.

Rabbit serum 1:10.	Fresh guinea pig complement, 1:10.			
	0.1 c.c.	0.2 c.c.	0.3 c.c.	0.4 c.c.
521	ooo ooo ooo	+	+	+
482	ooo ooo ooo	+	+	+
244	ooo ooo ooo	+	+	+
498	ooo ooo ooo	+	+	+

The hemolytic unit used was the rabbit-sheep system so standardized that 0.1 of a cubic centimeter of fresh guinea pig serum gave complete hemolysis in twenty minutes, and 0.05 of a cubic centimeter complete hemolysis in one hour in the water bath at 37° C.

At this time compound antigens prepared from the fourteen strains were used instead of the eleven single antigens used on June 3. An attempt was made to pool the antigens and thus employ a

TABLE VII.

Antigen No.				
1	*S. viridans*	14 strains	In 0.8% saline	Heated to 60° C. for 30 min.
2	*S. viridans*	14 strains	In 0.8% saline	Heated to 60° C. for 30 min. Chilled, digested, filtered.
3	*S. viridans*	14 strains	In distilled water	Heated to 60° C. for 30 min.
4	*S. viridans*	14 strains	In distilled water	Heated to 60° C. for 30 min. Chilled, digested, filtered.
5	*M. albus*	1 strain	In 0.8% saline	Heated to 60° C. for 30 min.
6	*M. albus*	1 strain	In 0.8% saline	Heated to 60° C. for 30 min. Chilled, digested, filtered.
7	*M. albus*	1 strain	In distilled water	Heated to 60° C. for 30 min.
8	*M. albus*	1 strain	In distilled water	Heated to 60° C. for 30 min. Chilled, digested, filtered.

single antigen for the detection of infection by any one of several strains, as was done by Schwartz and McNeil for the gonococcus.

Four antigens were prepared from a mixture of fourteen strains of *S. viridans:* two of them in saline solution were heated to 60° C. for thirty minutes and one of these was chilled on ice over night, digested in the thermostat at 37° C. for twenty-four hours, and filtered through a Berkefeld filter; two of them in distilled water were heated to 60° C. for thirty minutes, and one of these was chilled, digested, and filtered. Similarly four antigens were prepared from the *M. albus* strain and standardized (tables VII and VIII).

TABLE VIII.

Dilution antigen No. June 19, 1913.	Undiluted.						Diluted 1:10.						Diluted 1:100.						Amount used for tests.
	0.9 c.c.	0.7 c.c.	0.5 c.c.	0.3 c.c.	0.2 c.c.	0.1 c.c.	0.9 c.c.	0.7 c.c.	0.5 c.c.	0.3 c.c.	0.2 c.c.	0.1 c.c.	0.9 c.c.	0.7 c.c.	0.5 c.c.	0.3 c.c.	0.2 c.c.	0.1 c.c.	
1					+ + o	o	o	+ + o	+	+	+	+	+	+	+	+	+	+	0.5 c.c., dilution 1:10.
2					+	o	o	+ o o	+	+	+	+	+	+	+	+	+	+	0.5 c.c., dilution 1:10.
3					o	o	o	o	o	o	+	+	+	+	+	+	+	+	0.2 c.c., dilution 1:10.
4	Contaminated, not used																		
5	o	o	+ + o	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	0.3 c.c., undiluted.
6	o	o	+ + o	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	0.3 c.c., undiluted.
7	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Not used.
8	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Not used.

Antigens 7 and 8 were tested as high as 1.8 cubic centimeters to 0.1 of a cubic centimeter of serum against rabbit 498 which had been immunized with *M. albus,* and complement fixation was not obtained; antigens 7 and 8, therefore, were not used, since in distilled water chilling and digesting had resulted in no extraction of active antigenic fixable substance. The results were negative and it was found impossible to employ compound antigens 1, 2, and 5. Later the tests were repeated (table IX).

On June 25, seven days after the fourth and last inoculations, the rabbit sera were again tested against the compound polyvalent antigens, 1, 2, 3, and antigens 5 and 6 (table IX). The compound antigens were again found to be worthless.

TABLE IX.

Rabbit No. June 25, 1913.	*S. viridans* compound antigen.			*M. albus.*	
	1	2	3	5	6
521	+ + + + + o + + +	+	+	Antigen inhibits	+
482		ooo + oo + + +	+ + + + + o + + +	Antigen inhibits	+ + o + + o + + +
498 *M. albus*	+ oo + + + + + +	+	+	Antigen inhibits	o

On July 10, twenty-two days after the last inoculations, the rabbit sera were tested against single antigens prepared from eleven of the strains used for immunization; the results are shown in table X.

TABLE X.

Rabbit serum, July 10, 1913.	Wassermann reaction.	Gonococcus.	Antigens, *S. viridans.*										
			1	2	3	4	5	6	7	8	9	10	11
S. viridans 521	+	+	o	Antigen inhibits	o	o	Antigen inhibits	o	o	o	o	o	+ oo + + o + + o
S. viridans 482	+	+	o	Antigen inhibits	o	o	Antigen inhibits	o	o	o	o	o	ooo + oo + + o
S. viridans 244	+	+	o	Antigen inhibits	o	o	Antigen inhibits	o	o	o	o	o	o
M. albus 498	+	+	+	Antigen inhibits	+	+	Antigen inhibits	+	+	+ + + + + o + oo	+ oo + oo + oo	+ + o + + o + + +	+

SUMMARY.

1. *Streptococcus viridans* is of extremely low virulence for white mice and rabbits.

2. The serum of rabbits, after a series of intravenous injections of dead or living *Streptococcus viridans,* is poor in complement

fixative substance (*fixateur* of Besredka) for *Streptococcus viridans.*

3. Antigens prepared from several strains of *Streptococcus viridans* are relatively poor in fixable substance.

4. The technique of complement fixation tests for infection with *Streptococcus viridans* is so laborious and difficult that the method will not be available for general clinical work.

A NOTE UPON THE TECHNIQUE AND ACCURACY OF THE METHOD OF DOUGLAS AND HALDANE FOR CALCULATING THE DEAD SPACE IN BREATHING.*

By EDWARD PERKINS CARTER, M.D.

(*From The Saranac Laboratory for the Study of Tuberculosis, Saranac Lake.*)

PLATE 6.

The following notes are based upon the results obtained in a study of a group of cases of artificial pneumothorax, as employed in the treatment of pulmonary tuberculosis, of the application of the Haldane-Priestley[1] method of estimating the percentage of CO_2 in the alveolar air, and the calculation of the dead space by the method of Douglas and Haldane.[2]

No detailed report of these cases is undertaken at the present time, but in view of the recent critical analysis by Krogh and Lindhard[3, 4] of the method employed, it has seemed to the writer that certain observations, which have arisen in the course of this study, bearing upon the technique and accuracy of this method of determining the dead space, particularly in clinical cases, might be of interest.

No attempt has been made to compare the applicability of any of the other various methods of estimating the percentage of CO_2 in the alveolar air, or for the calculation of the dead space, as by Siebeck's[5] method, or by that recently described in Krogh and Lindhard's[6] basic paper.

* Received for publication, April 28, 1914.

1 Haldane, J. S., and Priestley, J. G., *Jour. Physiol.*, 1905, xxxii, 240.

2 Douglas, C. G., and Haldane, J. S., *idem*, 1912–13, xlv, 235.

3 Krogh and Lindhard, *Jour. Physiol.*, 1913–14, xlvii, 30.

4 Krogh and Lindhard, *ibid.*, p. 431.

5 Siebeck, R., *Skand. Arch. f. Physiol.*, 1911, xxv, 87.

6 Krogh and Lindhard, *loc. cit.*, p. 431.

Under the circumstances, any method involving a technique which must be carried out wholly in the laboratory was obviously impossible, as the cases studied were scattered about the village of Saranac Lake, many of them being confined to bed and only a small number being ambulatory. Fortunately the conditions under which it was necessary to carry on the observations fulfilled, in a large measure, the chief indication considered fundamental as affecting the accuracy of the Haldane-Priestley method of determining the percentage of CO_2 in the alveolar air, *i. e.,* the patients were at rest.

Krogh and Lindhard[7] have shown that the direct method of determining the composition of the alveolar air from samples taken by the Haldane-Priestley method becomes untrustworthy during muscular work, and in their later paper[8] they have emphasized this fact more fully. They also state:

> "During rest the increase in CO_2 percentage in the alveoli is not proportional to the time after the beginning of expiration, and the average of correct H.-P. inspiration and expiration samples is therefore *not* the true average tension. The difference can not be considerable, however, and we believe that in persons with a shallow respiration the H.-P. method may frequently be used with advantage as the variations in the composition of the alveolar air during the respiratory cycle must be small. There is a certain danger, however, that the respiration preceding the taking of a sample may be unconsciously but systematically altered (usually shortened) by the subject and that may vitiate the results to a great extent."

The technique of the method of procedure in this study was evolved in the endeavor to secure as great an accuracy as possible under the conditions imposed. It is self evident that the samples of alveolar air and of expired air must be secured under absolutely the same conditions, and it was soon found that the observations must be made, as far as possible, at the same hour of the day.

All the analyses have been made with Haldane's standard apparatus,[9] the samples being obtained by means of Haldane's gas sampling tubes.

[7] Krogh and Lindhard, *loc. cit.,* p. 30.

[8] Krogh and Lindhard, *loc. cit.,* p. 443.

[9] The apparatus was supplied by Baird and Tatlock, Ltd., London.

ALVEOLAR AIR.

The samples of alveolar air were obtained by the method of Haldane and Priestley, by the use of, in the earlier observations, the simple respiration tube, as originally described, with a single outlet for the alveolar air sampling tube two and a half inches from the mouth end. This tube was made from ordinary hose, and in order to overcome its tendency to curve it was reinforced by a thin brass rod wired to the outside, thus securing an absolutely straight egress and ingress for respiration.

After breathing quietly in and out through the tube, the nose being held, for a sufficient length of time to secure the normal respiratory rhythm for the individual under observation, the samples were secured at the end of a forced expiration following a normal inspiration (sample 1), and again at the end of a forced expiration following a normal expiration (sample 2). At the end of the sharp forced expiration the mouthpiece was closed by the tongue, the individual signaling by closing both eyes the instant the mouth piece was occluded, and the sample of alveolar air was taken.

It was manifestly difficult to obtain always the intelligent coöperation of the individual at the first observation, but as the observations were repeated this difficulty was overcome and the incidental error largely, if not entirely eliminated.

After samples 1 and 2 were secured, as soon as the normal respiratory balance was recovered the sample of expired air was obtained, originally by the use of Zuntz's[10] apparatus and subsequently by the use of rubber bags.

The average of the percentage of CO_2 in samples 1 and 2 was taken as the mean percentage of CO_2 in the alveolar air. It soon became evident, from a number of observations, that not infrequently the percentage of CO_2 in the alveolar air, as determined in this way (the mean of samples 1 and 2), if taken again immediately after collecting the expired air varied slightly from the percentage obtained previously. This difference was not always marked, but the percentage of CO_2 in the alveolar air taken immediately after

[10] This apparatus, as used by the writer, was made under the supervision of Dr. J. J. R. Macleod, in the Physiological Laboratory of Western Reserve University.

securing the expired air was more often found to be lower rather than higher.

Two possible explanations for this difference appeared reasonable. Inasmuch as we were dealing with individuals in whom any unusual though slight effort tended to produce a certain degree of dyspnea, it was conceivable that following the collection of the expired air the CO_2 percentage in the alveolar air was actually lowered; or it may well be that the difference between the two samples is the result of an unavoidable error in technique due to a difference in the time of the expiratory phase at which the sample was secured.

Using a four-way mouthpiece (figure 1),[11] the writer found that, in a sharp forcible expiration, the percentage of CO_2 in the alveolar air increased gradually, from that period of time as nearly as possible coincident with the end of the normal expiratory phase, up to the extreme limit of forced expiration, as follows:

	I. Percentage of CO_2.	II Percentage of CO_2.	III. Percentage of CO_2.	IV. Percentage of CO_2.
E. P. C.	4.84	4.87	5.29	5.62
E. P. C.	4.79	5.18	5.56	5.71
E. P. C.	4.31	4.71	5.41	5.59

I = approximately just after the limit of normal expiration.
IV = approximately the limit of forced expiration.

As a result of these observations the procedure was adopted of securing, in every instance, the two samples of alveolar air both before and after collecting the expired air. By means of the four-way mouthpiece samples 1 and 2 were secured, the expired air was then collected, and without any unnecessary delay the two additional samples (3 and 4) of alveolar air were obtained, and the mean percentage of CO_2 in all four samples was taken as the average percentage of CO_2 in the alveolar air at the time of the observation (table I).

[11] This mouthpiece has four outlets placed two and a half inches from the mouth end. The sampling tubes were opened and closed by colleagues in the order 1, 2, 3, and 4, at a given signal. Other similar records are not quoted. It is, of course, apparent that the figures 1, 2, 3, and 4 do not indicate with any accuracy the same period of time in the forced expiration in the different observations; they can be only approximately correct. (Krogh and Lindhard, *loc. cit.*, p. 431. This article was not published at the time the above observations were originally made.)

TABLE I.

Case No.	Date.	Tidal air in c.c.	Alveolar air CO_2% before taking expired air.	Alveolar air CO_2% after taking expired air.	Alveolar air CO_2, mean percentage.	Alveolar air CO_2 tension. In percentage of 1 atmosphere.	Alveolar air CO_2 tension. In mm. of mercury.
1	Jan. 15, 1914	294.3	5.73	5.51	5.62	5.61	40.1[12]
1	Jan. 19, 1914	174.6	6.02	5.98	6.00	5.65	40.4
1	Feb. 27, 1914	212.0	5.79	6.10	5.94	5.59	40.0
1	Feb. 28, 1914	281.0	6.16	5.63	5.89	5.57	39.8
1	Mar. 6, 1914	255.2	6.57	6.23	6.40	6.02	43.1
1	Mar. 7, 1914	261.0	6.55	6.26	6.40	6.02	43.1
3	Feb. 2, 1914	666.6	6.96	6.74	6.85	6.45	46.1
3	Feb. 3, 1914	728.7	7.02	6.65	6.83	6.43	46.0
3	Feb. 4, 1914	638.4	6.52	6.35	6.43	6.05	43.3
4	Feb. 18, 1914	534.2	6.16	5.82	5.99	5.64	40.3
4	Feb. 18, 1914	525.5	6.03	5.71	5.87	5.53	39.5
6	Mar. 11, 1914	569.0	5.39	5.52	5.45	5.13	36.7
6	Mar. 12, 1914	603.0	5.55	5.72	5.63	5.30	37.9

It is, of course, evident that such a four-way mouthpiece is not in any sense an essential necessity in securing the four samples. The writer adopted its use, however, as offering the greatest accuracy in standardizing his technique in the method of procedure, in avoiding any unnecessary delay after the observation was begun, and as a great convenience with bed patients. It has been used in all recorded observations since the end of January.

TABLE II.

Case No.	Date.	Tidal air.	I. Alveolar air CO_2%.	II. Alveolar air CO_2%.	III. Alveolar air CO_2%.	IV. Alveolar air CO_2%.	Alveolar air, mean CO_2%.	Alveolar air CO_2 tension. In percentage of 1 atmosphere.	Alveolar air CO_2 tension. In mm. of mercury.
9	Mar. 31, 1914	520.7	5.40	5.51	5.09	5.34	5.33	5.02	35.9
9	Apr. 1, 1914	542.0	5.39	5.59	5.09	5.41	5.37	5.06	36.2
9	Apr. 2, 1914	602.0	5.59	5.76	5.30	5.72	5.59	5.26	37.6
Mean		554.9	5.46	5.62	5.16	5.49	5.43	5.11	36.5

I = alveolar air sample following inspiration before collecting the expired air.
II = alveolar air sample following expiration before collecting the expired air.
III = alveolar air sample following inspiration after collecting the expired air.
IV = alveolar air sample following expiration after collecting the expired air.

[12] Saranac Lake is 503 meters above sea level. In all calculations the mean barometric pressure,—based upon actual barometric observations extending over three months,—is taken at 716 mm. of mercury.

As illustrating in greater detail the point emphasized, a single example will suffice (table II), being the results of three observations on successive days before the establishment of an artificial pneumothorax.

THE EXPIRED AIR.

The collection of the sample of the expired air was at first attempted by means of the Zuntz apparatus, in the hope that it would be possible to collect data regarding the respiratory quotient, which would be more reliable than when the bag is used, but this method was given up in favor of the rubber bag, as used by Douglas and Haldane[13] and their associates, for the following reasons. In a number of individuals the sense of air-hunger induced by the Zuntz apparatus was so great that the observation had to be abandoned in a very short time. It was also found that with the Zuntz apparatus the length of time necessary to secure an accurate sample of the expired air was longer than any of the cases studied could submit to; and finally there remained the difficulty of transporting the apparatus and meter from house to house, and its inapplicability for patients in bed.

With the rubber bag it is only necessary to collect the expired air for a definite length of time, counting accurately the number of respirations during the period of observation, and then to pass the amount collected through the wet gas meter, collecting the samples into previously exhausted sampling tubes. In this way one can collect any number of samples desired. Four minutes were taken as the standard length of time for the collection of the expired air. In a number of observations this was lengthened to five minutes, and in a few instances it was found necessary to shorten the time to three minutes, but in no case did the time fall below this latter figure. In individuals with a large tidal air the duration of any such observation depends in a measure upon the capacity of the bag employed. The two bags used had a capacity of fifty-two and fifty-seven cubic liters respectively.

The bag is used with an inlet tube of wide caliber about two meters long. By constructing a valve, on the principle of the in-

[13] Douglas, C. G., Haldane, J. S., Henderson, Y., and Schneider, E. C., *Phil. Tr. Roy. Soc. London,* 1913, cciii, series B, 185–318.

take valve of the Zuntz apparatus, and using dogs' intestine as the valve proper, it was possible to provide a valve which offers the least resistance to expiration and yet effectually prevents any unconscious rebreathing or escape of the air from the bag before it is clamped off at the conclusion of the observation. The allowance for dead space when measuring the expired air is ninety cubic centimeters.

Before starting the observation the patient breathes quietly into the bag for one to two minutes, without the valve in place, and this expired air is then forced out of the bag, thus assuring that the slight amount of residual air in the bag at the commencement of the observation was the individual's own expired air.

The method of procedure was as follows: At a given signal the patient starts breathing directly into the bag through the mouthpiece of the valve described above, inspiring through the nostrils and expiring through the mouth, the nostrils being held closed during expiration; the patients were also taught to occlude the opening in the mouthpiece with the tongue during inspiration, thus automatically reinforcing the valve.

In cases 3, 4, and 9 of this series, from which a few observations reported herewith are taken, it was found impossible to occlude the nostrils during expiration, because of the sense of discomfort produced, and the failure to do this leaves the method as employed in these cases open to a certain just criticism. A number of control observations, however, with different individuals, gave a difference of less than fifteen cubic centimeters in the tidal air figure when the expired air is collected in this way, at the same respiratory rate and for the same length of time, and no difference in its analysis.

In every instance the expired air was measured within half an hour after its collection.

In using the rubber bag two precautions are necessary and of fundamental importance as affecting the accuracy of the results obtained. Before measuring the expired air the bag must be thoroughly shaken to ensure a complete mixture of its contents, otherwise the resulting analyses of the CO_2 and O_2 may be wholly inaccurate. Finally, but by no means the least important procedure in the technique of the rubber bag method, the bag should be thor-

TABLE III.

Case No.	Date.	Respirations per minute.	Tidal air in c.c.	Alveolar air CO_2%.	Alveolar air CO_2 tension. In percentage of 1 atmosphere.	Alveolar air CO_2 tension. In mm. of mercury.	Expired air CO_2%.	Alveolar air in c.c.	Calculated dead space in c.c.	Remarks.
3	Feb. 3, 1914	17.6	729	6.83	6.43	46.0	4.24	465	264	Ambulatory, all observations sitting.
3	Feb. 4, 1914	17.5	638	6.43	6.05	43.3	4.32	428	210	
3	Mar. 20, 1914	15.0	508	6.78	6.37	45.6	4.29	327	187	
4	Feb. 4, 1914	14.0	500	6.09	5.72	40.9	4.18	343	157	Ambulatory, all observations sitting.
4	Feb. 4, 1914	15.0	551	6.19	5.83	41.6	4.27	380	171[14]	
4	Feb. 18, 1914	13.5	534	5.99	5.64	40.3	4.12	367	167	
4	Feb. 18, 1914	13.0	525	5.87	5.53	39.5	4.37	390	135[14]	
6	Feb. 11, 1914	15.4	423	5.87	5.53	39.5	4.08	327	96	Ambulatory, all observations sitting.
6	Feb. 11, 1914	18.7	413	5.21	4.90	35.0	4.12	332	81[14]	
6	Feb. 21, 1914	17.0	211	5.47	5.15	36.8	4.07	157	54	
6	Feb. 21, 1914	18.3	251	5.55	5.22	37.3	4.06	183	68[14]	
6	Mar. 3, 1914	18.3	396	5.34	5.03	36.0	4.04	301	95	
6	Mar. 3, 1914	18.3	328	5.60	5.27	37.7	4.14	242	86[14]	
9	Mar. 31, 1914	12.2	521	5.33	5.02	35.9	3.98	388	133	In bed.
9	Apr. 1, 1914	12.0	542	5.37	5.06	36.2	3.94	399	143	In bed.
9	Apr. 2, 1914	12.2	602	5.59	5.26	37.6	3.91	421	181	In bed.
9	Apr. 3, 1914	13.5	277	5.68	5.35	38.2	4.42	212	65	24 hrs. after first artificial pneumothorax.
10	Apr. 6, 1914	19.7	355	6.18	5.82	41.6	3.81	220	135	In bed.
10	Apr. 7, 1914	24.2	299	6.10	5.74	41.0	3.77	184	115	24 hrs. after first artificial pneumothorax.

[14] One hour after paracentesis for reëstablishment of artificial pneumothorax.

oughly washed out after each observation by ordinary atmospheric air, in order to prevent any accumulation of organic exhalations or the collection of pockets of CO_2 which may also give rise to inaccurate readings. This has been done by filling the bags from an ordinary laboratory bellows blower twice and then emptying completely. The writer found that unless this procedure was carried out the CO_2 percentages were not as accurate as when controlled by a control bag.

The figures in table III are taken from five of the cases studied in this series, and are chosen as being typically characteristic of the results obtained in an attempt to apply the method of Douglas and Haldane for determining the dead space in breathing in a group of individuals who fulfill to an unusual degree the requirement of bodily inactivity.

No detailed analysis of these cases is attempted at the present time; they are cited merely to add force to the points noted above in the technique of the method employed, and serve to emphasize the truth of Krogh and Lindhard's contention that:

"When however the alveolar CO_2 percentage is used together with the corresponding percentage in the expired air for calculating the dead space of the subject, even small deviations from the true composition of the air expired from the alveoli may have a very considerable effect upon the reliability of the result."

It must be borne in mind that all the individuals in this series are the subjects of a more or less advanced pulmonary tuberculosis, E. P. C. alone being entirely free from the disease. This fact explains the high percentage of CO_2 in the expired air.[15]

TABLE IV.

Four Control Observations Made on E. P. C. at Weekly Intervals.[16]

Respirations per minute.	Tidal air in c.c.	Alveolar CO_2%.	Alveolar air CO_2 tension.		Expired air CO_2%.	Alveolar air in c.c.	Calculated dead space in c.c.
			In percentage of 1 atmosphere.	In mm. of mercury.			
17	783	5.62	5.29	38.0	3.55	496	287
16	934	5.59	5.26	37.6	3.22	538	396
17	753	5.68	5.35	38.3	3.29	429	324
17	751	5.54	5.21	37.3	3.27	443	308

15 The actual analyses were all carefully checked by duplicates.

16 The writer has a large tidal air capacity, and a very constant respiratory rate. It is perhaps unnecessary to add that all the observations have been made on adults.

Turning to the figures given in tables III and IV, it is evident that in those instances in which the tidal air exceeds 423 cubic centimeters the calculated dead space is out of all true proportion to the alveolar air. In only one instance, observation 4, case 4, in which the tidal air is above this figure, does the calculated dead space for the individual approach what is probably the normal or true dead space for the individual at rest, and here the CO_2 percentage in the expired air rose abruptly following the paracentesis for the reëstablishment of the pneumothorax.

I wish, in concluding, to express my great indebtedness to Dr. Edward R. Baldwin, without whose interest and aid these observations would have been impossible, as they have been made almost entirely upon his private cases. To Dr. J. J. R. Macleod, of Western Reserve University, to whom I owe my introduction into the method of the physiological technique, my appreciative thanks are due. The study from which these brief notes are taken has been carried out entirely under Dr. Macleod's guidance. I am under deep obligations to him for many valuable suggestions and his friendly interest in the problems encountered.

CONCLUSIONS.

The determination of the percentage of CO_2 in the alveolar air, by the method of Haldane and Priestley, is sufficiently accurate for clinical purposes when the individual is at rest.

It is evident, however, that an error may arise in the determination of the percentage of CO_2 in the alveolar air, due to the time during the forced expiration, short of the extreme limit, at which the sample may be unconsciously taken. This error can only be overcome by taking several samples.

In individuals at rest having a tidal air below 425 cubic centimeters, the method of Douglas and Haldane for the calculation of the dead space gives approximately accurate results; with a tidal air above 425 cubic centimeters the results obtained may be wholly inaccurate.

In patients with marked pulmonary tuberculosis so slight an effort as that involved in collecting the expired air tends to lower the percentage of CO_2 in the alveolar air.

In collecting the expired air by the rubber bag method, it is absolutely essential that the bag be washed out with ordinary atmospheric air after every observation.

EXPLANATION OF PLATE 6.

Fig. 1. The four-way mouthpiece used in the experiments. For further details, see footnote 11, page 84.

STUDIES IN EXPERIMENTAL ATHEROSCLEROSIS.

A Preliminary Report.*

By I. ADLER, M.D.

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Plates 7 to 10.

In the present communication the protocols of the experiments will not be reproduced in detail, nor will the extensive literature that has gathered about the problems of atherosclerosis be discussed; and lastly, definite conclusions as to the result of the experiments will not be warranted. It is reserved for a future communication, when experiments still under way will have been concluded, and more definite knowledge as to the histological and chemical results may have been obtained, to report more in detail and with a critical appreciation of the literature.

All the investigations here reported have been done on dogs. The spontaneous occurrence of atherosclerosis in dogs, its histological structure, and the question of its greater or less analogy to human atherosclerosis are problems that have not received the attention that they deserve. In the text-books on animal pathology, little is to be found on the subject. Until recently the general opinion appears to have been that dogs were not disposed to atherosclerosis, and that if it occurred at all it was exceptional. Latterly it has been discovered that dogs are indeed subject to spontaneous atherosclerosis. The process is said to be limited, however, to the large vessels. The early stages appear as round or more oval or lengthwise patches of a whitish or pale yellowish and pinkish color, and are somewhat elevated above the surface of the intima. These sclerotic areas, it is said, are met with principally in localizations where the blood is apt to impinge with greater force against the wall of the vessel; for example, places where the vessel takes a sudden

* Received for publication, May 4, 1914.

turn in a new direction, or where other vessels branch off from the main trunk. The later stage of the atherosclerotic process is described in text-books as showing atrophy of intima and media, extensive fatty degeneration and calcification.[1] Della Vida[2] has seen spontaneous atherosclerosis in a large percentage of old dogs.[3]

Notwithstanding, however, that up to the present our knowledge of atherosclerotic processes in dogs is scant and insufficient, there seems to be no reasonable doubt that a process in many respects analogous to human atherosclerosis does occur. Granting this, for many years the writer has believed, and quite independently of experiments on dogs published in recent years, that if the possibility existed of producing experimentally a process that resembled in any way human atherosclerosis, one should be able to produce it in dogs, and not alone in rabbits. As the dog is omnivorous, it might be assumed, although it does not necessarily follow, that the metabolic conditions would approach more nearly those of human beings than is the case in the herbivorous rabbit. It is also an advantage that the disturbing median necrosis and calcification to which the aorta of the rabbit is readily disposed, is negligible in experiments on dogs. It is as yet not possible to state with certitude whether the structure of the aorta of dogs resembles the human aorta more closely than does that of the rabbit.

Much has been said and written concerning high blood pressure; various intoxications, such as lead and nicotin, and also infections with bacteria and bacterial products are important factors in the etiology of atherosclerosis. It was proposed to investigate some of these questions experimentally.

The first series of experiments was done during the greater part of 1908 and the first half of 1909.[4]

In this entire series the material to be tested was applied parenter-

[1] Hutyra, F., and Marek, J., Specielle Pathologie und Therapie der Haustiere, 3d edition, Jena, 1910, ii, 1100. Lyding, H., Zur Kenntnis der Arteriosklerose bei Haustieren, *Ztschr. f. Thiermed.*, 1907, xi, 359.

[2] Della Vida, M. L., Ueber experimentelle Arteriosklerose, *Deutsch. med. Wchnschr.*, 1913, xxxix, 2200.

[3] Celli, A., Manuale dell' igienista, 4th edition, Turin, 1912, ii, pt. 2, 1148.

[4] The writer wishes to express his thanks to Dr. William H. Park, through whose courtesy and interest it was possible to carry on this work in the laboratories of the New York Department of Health.

ally. The injections were supposed to be made into the jugular vein, with all proper regard to asepsis. It is not always easy to get the needle into the jugular vein, and it probably happened very often that the injection became subcutaneous instead of intravenous.

LEAD.

Former experience with rabbits has shown that the ordinary soluble lead salts were unsuited for hypodermic application that was to extend over any considerable length of time. The local reactions in every case were such as soon to render the injections impossible. It was found, however, that the triethylacetate of lead, as employed by Harnack[5] in his experiments, served our purpose admirably. Dr. Robert Emerson, then of Boston, prepared the salt in perfect purity. There was little or no local reaction, and the injections could thus be carried on *ad libitum.*

It should be mentioned that the blood pressure of our dogs was not tested, but that Harnack, contrary to the generally accepted opinion, found that in his animals the lead injections caused the blood pressure to rise. Two dogs were tested.

Dog 1.—The injections were commenced on Apr. 3, 1908. The animal was found dead in his cage on May 26, 1908. The symptoms corresponded closely to those described by Harnack. The temperature ranged from 101° to 104 3/5° F. There was considerable excitement, diarrhea, towards the end of the experiment bloody stools, and paralysis of hind legs. During the 55 days of the experiment the dog received injections amounting to 290 minims of a 1 per cent. solution.

Dog 2.—From June 1, 1908, to Aug. 18, 1908, when the dog was found dead. The symptoms were the same as in the first dog. The total amount injected was 590 minims. In both dogs no lesion could be found in the aorta or any other large vessel.[6]

NICOTIN.

Dog 1.—The experiment lasted from May 4 to Dec. 6, 1908, a period of about 7 months. The dog was found dead in the cage. 15 minims of a 1 per cent. solution of nicotin were injected at varying intervals, according to the condition of the dog, mostly every 2 or 3 days. There were never any convulsions. No lesions were present in the arteries.

[5] Harnack, E., Über die Wirkung des Bleis auf den thierischen Organismus, *Arch. f. exper. Path. u. Pharmakol.,* 1878, ix, 152.

[6] Harnack also failed to find any lesion in the vascular system of his dogs.

DIPHTHERIA TOXIN.[7]

Dog 1.—From May 12 to July 18, 1908,—about 67 days. The solution used was 1 : 100. Commencing with a single drop, the quantity injected was gradually increased until 1 c.c. was injected, after which the dog was found dead in the cage. No sclerotic lesions were present in any of the vessels.

Dog 2.—From Apr. 4 to Apr. 26, 1908. Death after 22 days. Temperature up to 104 1/5° F. One drop of the toxin was injected; twice only 2 drops. The result as to the arteries was negative.

Dog 3.—From Mar. 17 to Apr. 9, 1909. The solution used was 1 : 150. 1 to 10 drops were injected. Death in about 3 weeks. Temperature up to 104 1/4° F. Negative result.

EXPERIMENTS WITH BACTERIA.[8]

For the success of these experiments it was necessary to keep the animals alive as long as possible, but in a condition of mild sepsis. For this reason cultures of but moderate virulence were employed.

STAPHYLOCOCCUS AUREUS.

Dog 1.—Experiments commenced Mar. 10, 1908, and the dog was killed on June 13, 1908, after about 3 months. The injections, commencing with a single loop, suspended in normal saline, were gradually increased to the injection of an entire agar slant. The temperatures ranged from 100° to 104 2/5° F., showing the characteristic septic temperature curve. Result as to blood vessels negative.

Dog 2.—Oct. 30, 1908, to Mar. 15, 1909. Animal found dead after about 4 1/2 months. In this case the cultures were not so virulent as in the former experiment, and the sepsis was therefore not so marked, though nearly always an entire slant was injected. Temperature from 101° to 103 4/5° F. No arterial lesion.

Saltykow reported some time ago that he had obtained positive results from the injection of staphylococcus in rabbits. In his most recent publication,[9] however, he is inclined to attribute the positive result not to the injections of staphylococcus, but to the milk with which the rabbits were fed.

STREPTOCOCCUS PYOGENES ALBUS.

Dog 1.—From Oct. 30, 1908, to death, a little over 5 months, on Apr. 9, 1909. Cultures were not very virulent. Whole agar slants were used. Temperature varied in the neighborhood of 103° F. Result as to arteries negative.

[7] The diphtheria toxin was procured from the laboratories of the New York Department of Health.

[8] The cultures were obtained from the Board of Health laboratories, and were of various strains. I am also indebted to Dr. Alfred F. Hess, who controlled the purity of the cultures.

[9] Saltykow, S., Experimentelle Atherosklerose, *Beitr. z. path. Anat. u. z. allg. Path.*, 1914, lvii, 415.

COLON BACILLUS.

Dog 1.—From Mar. 10, 1908, to Oct. 15, 1908. Animal killed after about 7 months. Temperature ranged between 99 3/5° and 103 2/5° F. Animal gained weight during the experiment. Arteries normal.

Dog 2.—Oct. 30, 1908. Found dead after 5¾ months, on Apr. 17, 1909. The animal had developed a number of abscesses. Temperature ranged from 102° to 104° F. Arteries normal.

TYPHOID BACILLUS.

Dog 1.—Oct. 30, 1908. Killed after about 6 months, on Apr. 30, 1909. Temperature ranged from 101 1/2° to 103 1/5° F. Profuse diarrhea and frequent bloody stools. Arteries without lesion.

I was compelled to interrupt these studies for a number of years, but was enabled to take them up again in January, 1913.[10]

It has been shown in former experiments that the injection of adrenalin alone seemed to have no effect upon the arteries of the dog.[11] It was now proposed to test the effect of the high blood pressure caused by the adrenalin, combined with severe bodily exertion, with special reference to the purely mechanical "*Abnutzungstheorie*" that had at one time been generally and favorably received. For this purpose, a treadmill was constructed, so that two dogs could run on it simultaneously and at the same speed, and could not stand still during the period of exercise; they were, moreover, so harnessed that they could not lie down. One dog was injected with gradually increasing doses of adrenalin 1 to 1,000, usually every other day, sometimes several days in succession. It was intended to inject the adrenalin into the jugular vein, but the injections were probably mostly subcutaneous.[12]

The second dog served as a control; it received no injection, but worked steadily together with the other dog in the treadmill. The

[10] I wish to express here my grateful obligation to Professor Frederic S. Lee, Director of the Physiological Department of the College of Physicians and Surgeons of Columbia University, for placing the conveniences of his laboratory at my disposal.

[11] Otto, C., Über Arteriosklerose bei Tieren und ihr Verhältnis zur menschlichen Arteriosklerose, *Virchows Arch. f. path. Anat.*, 1911, cciii, 352. Otto finds that dogs are much less sensitive to adrenalin than rabbits, but claims some positive results in the dogs' aortas after the injection of astoundingly large quantities of adrenalin.

[12] I am indebted to Dr. B. S. Oppenheimer for carrying out the injections and for performing the autopsies on both dogs.

experiment was started on January 20, 1913. Running in the treadmill began with twenty-five minutes in the morning and thirty minutes in the afternoon. Occasionally for a day or two there was no running, but on the whole the work went on fairly steadily, and was increased gradually so that at the end of the experiment, on June 2, 1913, when both dogs were killed, they ran at top speed for almost four hours in the morning and about three hours in the afternoon. The adrenalin injections began with ten minims, and the dose was gradually increased so that at the end of about four months the dog received eighty minims at a single injection, the total amount injected in sixty injections during 130 days being 1.676 grams. These doses are minute compared to the huge quantities injected by Otto,[13] who injected 49.5 grams of pure adrenalin in the course of five months in seventy-eight injections. The initial weight of the adrenalin dog was 11,870 grams; he gradually gained in weight, so that on April 4 he reached his highest weight of 15,300 grams; from then on he gradually lost again, so that at death he had again come down to about his original weight. The control dog weighed 10,220 grams, on March 16 had gained about 2 kilos, and at death weighed about 8,780 grams. A very careful study of the blood vessels after death failed to show any arterial lesion in either dog.

It was soon noticed that, while the adrenalin dog was active and happy, and was practically untiring in running the treadmill, and always at top speed, the control dog was very soon tired, dispirited, and at the end of his forced run completely played out. It is worth noting, also, that the adrenalin dog developed no glycosuria. These facts correspond with Cannon's recently expressed views.

The uniformly negative result of all these experiments with reference to the arteries was calculated to impress one very strongly with the conviction that mere mechanical conditions, as well as intoxications and bacterial infections of various kinds, were not, at least in the dog, determining factors, and that parenteral methods were not likely to lead to any results. In the meantime, moreover, the researches of Aschoff, Anitschkow, Chalatow, Ignatowsky, Saltykow, Steinbiss, and others had been published, from which it appeared that lesions resembling human atherosclerosis very closely

[13] Otto, C., *loc. cit.*

could be produced principally in rabbits, by feeding these animals on proteins and lipoids, especially cholesterin. Following along these lines, and on the theory that atherosclerosis was probably caused by chemical rather than by mere mechanical influences, another set of experiments was undertaken to test the possible effects of various kinds of chemical substances added to the food of the animals.

From the fact that chondroitin sulphuric acid can be obtained in comparatively large quantities from the arteries, and especially the aorta, it was thought that adding this substance to the ordinary food of dogs might possibly give some results.[14]

The dog, a healthy male fox-terrier, was fed in the usual way with bread and meat. On Oct. 24, 1913, a capsule containing 0.5 gm. of the soda salt of chondroitin sulphuric acid was added to his daily meal until Nov. 25, 1913. No capsules were given until Dec. 6. After that 1 gm. of the salt was given daily until Dec. 30. The dog became ill with an abscess below the right ear, from which he recovered, and fed well most of the time. The capsules did not seem to affect him. The animal died on Jan. 18, 1914, nearly 3 months after the beginning of the experiment. His weight varied according to his general condition, decreasing when the animal was ill, increasing so that at one time he gained 1 1/4 kilos, and at his death weighing about the same as when the experiment started. The total quantity of chondroitin sulphuric acid salt consumed was 47 gm. The cholesterin content of the blood appeared to be a little below the normal, being 0.001, while the normal for dogs is about 0.00137.[15] The autopsy showed a normal aorta and large vessels.

It was now proposed to feed dogs on bread and cottonseed oil, either without any, or with only a very small quantity of protein in the food. It was assumed at first that the animals could consume about 200 cubic centimeters of oil daily, but it was soon found that about 50 cubic centimeters was all that they could manage and keep in fair condition. The low protein diet was probably not necessary.

Dog 1.—Experiment commenced Nov. 8, 1913. The oil was rapidly increased to 200 c.c. per day. For the first few days the dog ate the food. After that he would eat only one third of his daily meal, and at times he would not touch the food at all. He was killed in a fight with another dog on Dec. 6, having lost about a kilo in weight.

The cholesterin content of the blood was not determined. Autopsy showed normal vessels.

[14] I am indebted to Dr. P. A. Levene of The Rockefeller Institute for Medical Research for placing a great quantity of the chemically pure soda salt of chondroitin sulphuric acid at my disposal.

[15] Dr. Edwin Henes, Jr. (Untersuchungen über den Cholesteringehalt des menschlichen Blutes bei inneren Erkrankungen, *Deutsch. Arch. f. klin. Med.*, 1913, cxi, 122) kindly made the cholesterin determinations for me.

Dog 2.—Experiment commenced Dec. 11, 1913. Bread, 2 oz. of meat, and 150 c.c. of cottonseed oil. The dog ate all the food for 3 days, after which he sometimes took one third, one half, or sometimes the whole. On Jan. 10, 1914, the oil was reduced to 50 c.c. From then on the dog ate all that was given him, till death on Jan. 21, 1914. His weight remained about the same, till the end, when he lost about 1 kilo.

The cholesterin content was 0.00225, almost double the normal. The autopsy showed pneumonia and a duodenal ulcer. There were a number of slight fatty streaks in the aorta.

Dog 3.—Experiment commenced on Nov. 29, 1913. Bread, 2 oz. of meat, 150 c.c. of oil, which were reduced on Jan. 6 to 50 c.c. From Dec. 23 to Jan. 28 meat was taken away altogether; the dog ate bread and oil only. It was attempted to make him run in the treadmill; for a number of days he could be made to work for an hour or even more. He developed an abscess on the jaw, so that he could run only at irregular intervals. Then convulsions set in, and the running had to be stopped altogether. He was killed on Jan. 31, the experiment lasting about 2 months. The weight increased slightly at first, then decreased somewhat, but at death was about the same as at the beginning, notwithstanding attacks of vomiting and diarrhea, besides convulsions.

The cholesterin content of the blood was 0.00228, also markedly above the normal. The anatomical diagnosis and autopsy showed adherent pericardium, dilatation of heart, fatty deposits in the liver and kidneys, pulmonary emphysema, intestinal parasites, and fine fatty striæ in the aorta.

URANIUM NITRATE AND OIL.

The oil feeding was then combined with uranium nitrate.

On Nov. 19, 1913, a dog was injected subcutaneously with 1 c.c. of a solution of uranium nitrate 0.5 to 100. The injection was repeated the next day. Typical symptoms of severe uranium nephritis followed. He was fed with oil soon reduced to 50 c.c., bread, and 2 oz. of meat. On Nov. 6 meat was taken away entirely, and the dog ate well most of the time. On Jan. 11, 1914, meat was given again. The animal died on Feb. 12, 1914, nearly 3 months after the beginning of the experiment. He received altogether 6 c.c. of uranium nitrate solution in five injections. The urine at death had a specific gravity of 1.018, albumin 0.3 per cent. (Esbach), some red cells, and numerous casts, mostly hyaline. He lost steadily in weight, altogether about 2,580 gm.

The cholesterin content of the blood was low, being only 0.00013. The autopsy showed typical uranium nephritis and infarction of lung. There were no changes in the aorta or large vessels.

A second uranium oil dog died eighteen days after the beginning of the experiment, without showing any special lesions besides nephritis.

CHOLESTERIN.

On January 27, 1914, an experiment was commenced with feeding cholesterin.

Dog 1.—The dog received bread, meat, 50 c.c. of cottonseed oil, and every other day a capsule of 0.5 gm. of pure cholesterin (Merck). It was not proposed to give the large doses of cholesterin which other investigators have given to rabbits and dogs, the idea being not to go beyond normal conditions more than was absolutely necessary. The animal took his food well until about the middle of Feb.; at that time the oil was stopped, and from Feb. 25 on he took 0.5 gm. of cholesterin every day with his bread and meat. Repeated attempts to make him run on the treadmill failed, on account of his feet becoming sore after running a day or two. After about 2½ months from the beginning of the experiment, the animal died on Apr. 8, 1914. The original weight of 6,700 gm. decreased steadily, so that at death he weighed only 2,400 gm. The autopsy showed pneumonia in the lungs; there were numerous small but quite distinct fatty patches and striæ in the aorta. The total quantity of cholesterin consumed was 25 gm. The cholesterin content of the blood serum was 0.001808.

Dog 2.—Feb. 5, 1914. Bread, 2 oz. of meat, 50 c.c. of oil, and 0.5 gm. of cholesterin were given every other day. Oil was stopped after 12 days. From Feb. 25 on 0.5 gm. of cholesterin was given daily. The dog ate well from the time the oil was stopped, and was found dead on Mar. 19, 1914. His weight had decreased about 900 gm. During the 1½ months he had consumed 16 gm. of cholesterin. The cholesterin content of the blood serum was 0.00196. The autopsy showed ascites, probably due to nephritis; there was a small, cartilagenous patch in the sinus above the posterior aortic cusp, surrounded by a yellowish area of hypertrophied intima; there were besides a number of hypertrophic spots in the thoracic aorta. The bile in the gall bladder contained large quantities of doubly refracting cholesterin crystals.

HYDROCHLORIC ACID.

A casual remark of Dr. P. A. Levene suggested the simple procedure of adding dilute hydrochloric acid to the dog's food and thus producing a chronic hyperacidity.

Dog 1.—Beginning Oct. 24, 1913, at first 20, after a few days 40, then 50, and finally 60 drops of dilute muriatic acid were added to the dog's ordinary food of bread and meat. The animal took the acid without any difficulty, and fed well till the last few days before death, which occurred on Dec. 13, 1913, about 2 months after the beginning of the experiment. Though the dog took all his food and seemed perfectly well, he nevertheless lost 3 kilos. The cholesterin content of the blood was not made in this case. The autopsy showed faint yellowish streaks just above the sinuses, behind the pulmonary cusps. The aortic cusps were normal. In the abdominal aorta, about 4.5 cm. above bifurcation, several raised patches of pinkish yellowish color were seen, somewhat irregular in shape, and with the surface wrinkled, the long axis parallel to the long axis of the

vessel: there were some patches also around the orifices of efferent vessels. Transverse ridges are marked in some of these patches, especially in several that are situated in the internal iliacs. Some similar patches are also recognizable in the carotids. There was considerable edema of the left lung, but no pneumonia. The cause of death is uncertain.

Dog 2.—Beginning Dec. 22, 1913, dilute muriatic acid was added to the food, so that after 21 days the daily quantity was 60 drops, which after that was not increased. The dog was killed on Mar. 25, 1914, 3 months after the beginning of the experiment. The animal was well during the entire time, and ate greedily. He was not full grown when the experiment was started, but grew steadily, and had gained three kilos when killed. The cholesterin content of the blood serum was 0.00159, just a trifle above the normal. At autopsy the dog was found to be well nourished, with good adipose. The heart contained numerous *Filaria mitis;* the endocardium of the left ventricle was somewhat milky. No valvular lesions were present. Immediately above the aortic cusps in the sinuses of Valsalva, also near the origin of the left carotid, and especially in the abdominal aorta, and there again most pronounced in its lower part, there were greyish yellow patches slightly raised above the surface of the intima. The largest patch was situated near the origin of the innominate artery, and around this the aorta is decidedly thin. The iliac arteries showed distinct transverse striation with yellowish grey and slightly raised mottling. There was nothing abnormal in the carotid and renal arteries. Otherwise there were no gross lesions.

DISCUSSION.

In reviewing briefly the outcome of all the experiments thus far reported, one fact stands out prominently; namely, that all the injection experiments failed absolutely to yield any positive result. Neither the poisoning with nicotin or lead, nor the infections with bacteria or bacterial products, appeared to afford the slightest evidence of atherosclerotic lesion in the aorta, or in any other vessel or organ of the dogs treated. If reasoning by analogy be permitted, it may be assumed that if a bacterial infection in man, which rarely lasts longer than a few weeks, is supposed to be an important etiological factor in subsequent atherosclerosis, then bacterial infections in dogs, prolonged for many months, and finally ending in death, might reasonably be expected to cause some atherosclerotic change.

In the case of the two dogs running in the treadmill, the objection might be raised that, though the strength of the animals was taxed to the utmost, the experiment did not last long enough to cause any damage. This objection, however, loses much of its weight when one finds from the last group of experiments that positive results can be brought about in time equally short or even shorter.

This last group of experiments, though still far from concluded, has already apparently furnished some points of interest. Chondroitin sulphuric acid does not seem to be effective, and does not require further discussion. All the other experiments, with the exception of two dogs to whom dilute hydrochloric acid was given, were undertaken for the purpose of studying the now generally conceded causal relation between cholesterin and the atherosclerotic process in man, as well as in the rabbit. Pure cholesterin (Merck) was given only in two cases. The other dogs took cottonseed oil, not only because this was less expensive than cholesterin, but also because it was thought probable that this oil contained sufficient lipoid material to answer every purpose. It was not proposed to administer the colossal quantities of cholesterin which some investigators had employed, and which exceeded by far all that either man or the rabbit would ever consume under natural conditions.

In all the oil and cholesterin dogs, with but one exception, atherosclerotic lesions were found. It is true, however, that only the very earliest stages appeared; *i. e.,* the fine yellowish striæ, and the fatty patches, in their gross appearance closely resembling the same structures so familiar in the human aorta.

The one exception was the dog with uranium nephritis. On general principles one would have expected that an oil-fed dog with chronic uranium nephritis, would be much more likely to develop atherosclerosis than a dog with sound kidneys. This seems not to have been the case. Neither in the aorta, the renal artery, nor in the kidneys, in fact in none of the vessels or organs of the nephritic dog, could any trace of atherosclerosis be found. The cholesterin content of the blood serum in this dog was much lower than in any other dog. It will be the subject for further study to arrive at an understanding regarding these phenomena.

In the aorta of the first acid-fed dog, as described above, there were found not only the fine, fatty striæ in the thoracic aorta, but quite large raised sclerotic patches in the lower portion of the abdominal aorta and in the external iliacs. These patches closely resembled those atherosclerotic areas in human beings that have not yet become ulcerated or calcified. This dog, however, was about four years old. The obvious objection was therefore close at hand

that the atherosclerosis in his case might have been spontaneous and entirely independent of the acid feeding. A young and growing dog was therefore subjected to the same treatment, and for a somewhat longer time, with the result that atherosclerotic changes similar to those in the first dog were now manifest over a considerably greater area of the aorta, both thoracic and abdominal, but more pronounced in the abdominal aorta. Though only two dogs have thus far been fed with hydrochloric acid, the possibility can not be denied, especially in view of the numerous negative results with other methods, that these positive results are not mere coincidences, but are probably due to the hydrochloric acid. Further investigations are necessary, and are under way.[16]

While writing this communication, the work of Oswald Loeb[17] has come to my notice. He describes results similar to mine in two dogs which he fed with large doses of lactic acid and kept on an exceedingly low protein diet. The detailed reports of his experiments, and especially the microscopic findings, have not yet been published. No diminished protein diet was given to our dogs. They fed well on their usual diet of bread and meat, thus showing that the sclerotic affection of the large vessels was in all likelihood due to the acid, and that it was probably immaterial whether the dogs ate meat or not.

The histological structure of this canine atherosclerosis has not yet been completely studied in all its details, but it has been ascertained that the lesions are primarily and principally localized in the intima. The intima of the dog's aorta, however, appears to differ somewhat in its structure from that as found in man and in the rabbit. It would appear that under normal conditions the elastica interna is covered by a single layer, possibly by several, fine layers of endothelial cells. Fibrous or muscular tissue is nowhere perceptible. The very earliest beginning of the sclerotic process is marked by a proliferation of rather large, flat cells, in all probability of en-

[16] Since writing the above, I have examined a third dog. The animal, scarcely one year old, died of pneumonia after having had acid feeding only for 3 weeks He, too, showed a number of typical yellowish patches in the arch of the aorta.

[17] Loeb, O., Ueber experimentelle Arterienveränderungen mit besonderer Berücksichtigung der Wirkung der Milchsäure auf Grund eigener Versuche, *Deutsch. med. Wchnschr.*, 1913, xxxix, 1819.

dothelial origin. Notwithstanding protracted search, and aided by the best technical methods and all sorts of stains, nothing in any way resembling the macrophages or the large amebic leucocytes described by Saltykow could be found (figure 1). Almost simultaneously with the appearance of these large cells, elastic fibers begin to be split off from the elastica interna, and the whole process, as described by Jores, takes place (figure 2). A larger or smaller elevation or excrescence is thus formed, the base of which is still the elastica interna, and for some distance there can also be recognized one or two rows of those peculiar large endothelial-like cells, at the base of the elevation, and resting immediately upon the elastica. Between the newly split off elastic fibers which form, as it were, the skeleton of the little excrescence, there now develops a more or less swollen and succulent connective tissue, and possibly also some muscular fibers. In the meanwhile, and in that portion of the media underlying the newly formed elevated patch, the elastic fibers are beginning to give way, and the tissues there are also in a swollen and succulent state. No aggregation of leucocytes, or anything else pointing to an inflammatory process, could be made out. In some text-books it is stated that calcification has been found in the spontaneous atherosclerosis of dogs, and it is perhaps not improbable that calcification would soon have followed the developments just described, had the experiments lasted sufficiently long. Further investigation is required to clear up this point. It does not appear unreasonable to presume that the calcium metabolism in dogs, as well as in other omnivorous animals, differs from that of the rabbit, in which calcification, primarily in the media, occurs early and often spontaneously.

Simultaneously with the first appearance of the proliferating cells, and only among them and not in the normal intima, minute sudanophil droplets are seen contained in the upper portions of the proliferating cells, and also lying free above and between them. Very soon they are found in the media and down to the adventitia. As the process proceeds, the droplets become larger, sometimes run together, but never have we found them of the size and number as described in the rabbit and in man. They are most numerous in those fatty striæ and elevated fatty patches that we

found in the aortas of the oil-fed, and especially of the cholesterin-fed dogs (figure 3), but they also occur with great constancy, though much smaller in size, in the acid-fed dogs. They stain readily with Sudan III and Scharlach R; but they are optically inactive, and therefore are probably not cholesterin, but some other as yet undetermined fatty or lipoid substance. It is a striking fact that, while the bile of the cholesterin-fed dogs contained innumerable doubly refracting cholesterin crystals, not a single one could be found in the arteries, the liver, and the adrenals. Chalatow[18] has recently pointed out that the deposit of the doubly refracting so called fluid crystals, most abundant in the rabbit, does not take place in all animals. It would seem from the observations on our dogs, that, though the cholesterin content of the blood serum may be high, there is nevertheless no permanent deposit of cholesterin in the organs, and it is probable that there is a rapid elimination.

It is noteworthy, also, that the acid-fed dogs showed numerous sudanophil droplets in the early stages of the sclerotic hypertrophy; but that these sudanophil bodies had almost, if not entirely disappeared by the time the fully formed sclerotic area had developed.

The livers of all the dogs, as well as the adrenals, were crowded with sudanophil bodies. It could be shown that there was no fatty degeneration, but merely an overcrowding of the cells, and even to some extent of the intercellular tissue, with an infiltration of fatty material. Doubly refracting components could never be found. The livers of the acid-fed dogs, more than those of any of the other animals, were filled with this fatty substance, and it was especially the cylindrical epithelium of the bile ducts that was most conspicuously loaded with brilliant sudanophil matter (figure 4).

The work is being continued, and definite conclusions would at this stage be premature; but perhaps it may be permitted, even now, to venture the statement that in all probability the theory which bases atherosclerosis on a purely mechanical etiology will not prove tenable. Whether mechanical factors come into play at all, and if so, to what extent, remains to be seen.

It seems almost certain, at least in our present state of knowledge,

[18] Chalatow, S. S., Über flüssige Kristalle im tierischen Organismus, deren Entstehungsbedingungen und Eigenschaften, *Frankfurt. Ztschr. f. Path.*, 1913, xiii, 189.

that chemical influences, subject possibly to more or less nerve control, are dominant factors in the etiology of atherosclerosis. Perhaps it may be discovered also that cholesterin and its various modifications and combinations, while undoubtedly an element of importance in atherosclerosis of the rabbit and human beings, may not be the sole predominant etiological factor. If it should turn out that so simple a procedure as adding a certain proportion of hydrochloric acid to the food of dogs is sufficient to produce lesions of the blood vessels closely analogous, if not wholly identical with human atherosclerosis, a revision of our present theories will become necessary.

EXPLANATION OF PLATES.

Plate 7.

Fig. 1. Section through a sclerotic patch from the aorta of an acid-fed dog. Weigert-Van Gieson stain shows the proliferation of endothelial cells and the typical hypertrophy of the intima.

Plate 8.

Fig. 2. Section through the same aorta. Weigert elastic stain shows splitting off of elastic fibers from the elastica interna.

Plate 9.

Fig. 3. Frozen section through a yellow patch of the aorta of an oil-fed dog. Sudan III shows incipient proliferation of the endothelium and distribution of sudanophil bodies.

Plate 10.

Fig. 4. Frozen section through the liver of an acid-fed dog. Stained with Sudan III. The liver cells and especially the cells of the bile ducts are crowded with sudanophil bodies.

THE RELATION OF THE SPLEEN TO BLOOD DESTRUCTION AND REGENERATION AND TO HEMOLYTIC JAUNDICE.

X. Concerning the Supposed Regulatory Influence of the Spleen in the Formation and Destruction of Erythrocytes.

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In the older literature of the physiology of the spleen appear numerous references to differences in the composition of the blood of the splenic artery as contrasted with that of the splenic vein, and in the recent literature observations are made upon the occurrence of a specific hemolysin in extracts of splenic pulp. The possible bearing of these observations upon the various studies[1] of the spleen reported from this laboratory during the past two years led to a critical examination of the general literature and to certain experiments already described, in the hope that light might be thrown upon some of the difficult aspects of the general problem. A careful study showed that the observations in question are subject to a large experimental error which renders the finding of slight differences in the composition of arterial and venous blood of doubtful value,—a reason why the work has not heretofore been utilized in our reports. Indeed the work probably would have been entirely disregarded if Banti had not recently claimed, in support of his theory of the splenic origin of icterus, that the blood of the splenic vein normally contains more free hemoglobin than the blood of the general circulation. In order to test this and other points we have repeated experiments upon which the assumption that the spleen has a direct or indirect influence upon the red cells is based.

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[1] *Jour. Exper. Med.*, 1912, xvi, 363, 375, 758, 769, 780; 1913, xviii, 487, 494, 665; 1914, xx, 19.

Much of the early work on this subject is not only contradictory but was done before the development of the present exact methods of blood examination. Thus Virchow (1) found fewer red cells in the blood of the splenic vein than in that of the artery; while Malassez and Picard (2) and Emelianow (3) report the opposite. On the other hand, later investigators, Vulpius (4) and Paton, Gulland, and Fowler (5), have found no constant or noteworthy differences.

Considering the spleen as a possible leucoblastic organ, numerous early observers (6) found relatively more leucocytes, especially so called young forms, in the blood emerging from the spleen, than in that entering it. Tarchanoff and Swaen (7) and also Virchow (1) could not find any noteworthy difference, whereas Paton, Gulland, and Fowler (5) noted a constant diminution in the number of leucocytes in the splenic vein as compared with the general circulation. In this connection Bulgak (8), who describes an increase in leucocytes in the splenic vein, states that this is true of the venous blood of all parenchymatous organs. Freyer (9) concludes from his comparative counts that the spleen has nothing to do with blood formation.

The preceding studies refer of course to mature animals. It is accepted that in fetal life the spleen has the power of extensive blood formation, and several reports are at hand to show that the adult spleen may undergo, in the presence of injury to the bone marrow, a myeloid metaplasia (10); that is, that it can regain under pathological conditions its fetal function. Whether or not the spleen may exert this power of blood formation in the adult under normal conditions is doubtful, though still an open question.

Although the spleen certainly destroys red blood cells, as is evident from the presence in it of large cells, phagocytic for erythrocytes, which are increased under certain pathological circumstances, there still exists doubt whether the destruction by phagocytosis is the only method of red cell disintegration. It is stated also that the erythrocytes, in their passage through the spleen, are so acted upon by some unknown substance as to become more susceptible to hemolysis. This is the basis of Bottazzi's (11) hemocatatonistic theory, which has recently received support from Banti (12) and his colleague Furno (13). In the course of an investigation of hemolytic splenomegaly, they studied normal animals and those receiving hemolytic serum and came to the conclusion that free hemoglobin can be demonstrated in the blood of the splenic vein in normal animals as well as in animals receiving hemolytic serum. It was found at times in the blood of other vessels but in less amounts than in the splenic vein. They consider the findings as evidence of hemolysis in the spleen. The red blood cells of the splenic vein were found also to be less resistant to hypotonic salt solution than were those of the general circulation. Observations by Chalier and Charlet (14) on the resistance of red cells in the splenic artery and vein gave different results. Although the venous blood in general was slightly less resistant than arterial blood, this was reversed in the splenic system, so that the blood of the splenic vein was more resistant than that of the splenic artery and much more than the blood of other veins. Hammarsten is also said by Gabbi (15) to have found that the splenic vein blood was more resistant than the arterial. In the observations of Banti and Furno, the reference is to free hemoglobin in the serum and not to the increased hemoglobin content of venous or splenic blood described by several investigators (16).

The claim of Banti and Furno is surprising in that they state that the dissociated hemoglobin of the serum ("*emoglobin disciolta dal siero*") is not only always present in the splenic vein of normal animals, but in sufficient quantities sometimes to be measured by a Sahli hemoglobinometer. It is to these observations that we have given especial attention in our work.

COMPARISON OF THE ARTERIAL AND VENOUS BLOOD.

Method.—From dogs under ether anesthesia, blood was obtained directly from the splenic artery and the splenic vein. Great care was exercised to disturb the vessels and the organ as little as possible, as it has been shown by Grigorescu (6) and Pribram (17) that the cell content of the blood may be greatly increased by congestion of the spleen. From a nick in the vessel wall of one of the branches of the artery or vein, fresh blood was drawn directly into Thoma blood-counting pipettes and the capillary tube of a von Fleischl hemoglobinometer. From another branch, blood was withdrawn by a syringe and immediately distributed to tubes containing different strengths of hypotonic salt solution designed to test the resistance of the red cells. Some of the blood was also set aside for similar tests with washed cells. For the determination of the presence of free hemoglobin in the serum, blood was collected in three ways: (1) in a paraffined centrifuge tube, (2) in a tube containing potassium oxalate, and (3) by drawing it directly into tubes through capillary points which were then sealed. All three samples were then centrifuged and the serum was examined for hemoglobin by visual inspection and the spectroscope. Smears for differential counts were made at times from the blood flowing directly from the vessel and at times from a drop from the syringe. Finally, tests for reticulated or skeined (young) red blood cells were made. This was done by letting a few drops of blood fall into a solution of brilliant cresyl blue, and, after standing fifteen or twenty minutes, the skeined forms in proportion to the unskeined or mature forms were counted in fresh smears. For the purpose of controls, blood from the femoral vein, and from the capillary circulation by puncture of the skin was occasionally collected.

Results.—The figures for the red and white cells, differential counts, and total hemoglobin in a series of five dogs show that as far as these estimations are concerned the blood of the splenic vein does

not differ greatly from that of the artery. The variations are not uniformly on one side and are all within the limit of error inherent in the methods of blood examination.

It is of interest that in these and other dogs, the red cells of the vein, in six of eight animals, showed more or less marked anisocytosis and inequality of staining, which were not seen to the same degree in the blood of the artery. Polychromatophilia was about equal in artery and vein. In two of the eight animals a few normoblasts were found in the splenic vein blood only. Control smears from the femoral vein of four dogs showed changes in the red cells about equal to that of the splenic vein, indicating that these changes are characteristic of venous blood in general rather than a specific change caused by passage through the spleen.

In regard to the presence of free hemoglobin in the serum, if we had depended on one tube only, we should have occasionally found apparent hemoglobinemia, both of the general circulation and of the splenic vein; but as in every set of three tubes, in a series of seven dogs, at least one was free of hemoglobin, we cannot support the view that free hemoglobin in demonstrable amounts is present normally either in the splenic vein or in the general circulation of the dog. Our experience forces us to the conclusion that the findings of other investigators are due to hemolysis after collection or are dependent upon the method of separating the serum.

As regards the resistance of the red cells, of which comparative tests were made on eight dogs, in five no difference was found between artery and vein; in the other three, the venous corpuscles were slightly less resistant. Two control tests with cells from the femoral vein showed these to have the same resistance as those of the splenic vein.

In seven comparative tests for skeined or reticulated red corpuscles, these were found five times to be more abundant in the splenic vein and twice more numerous in the artery; the differences were never very striking. Five controls from the femoral vein corresponded more closely to the splenic artery counts than those of the splenic vein.

Conclusions.—As a result of the various observations we conclude that the slight differences between the arterial and venous

blood of the spleen are within the limits of error inherent in the methods of blood examination and are not to be explained by a peculiar action of the spleen. In some instances peculiarities shown by the splenic venous blood are common to the venous blood of the general circulation. Banti and Furno's observation concerning the presence of free hemoglobin in the blood of the splenic vein is not confirmed.

THE HEMOLYTIC POWER OF SPLENIC EXTRACTS.

The histological evidence of the destruction of erythrocytes by phagocytic cells of the spleen has naturally suggested the possibility of the liberation by these cells of a ferment capable of acting extracellularly. If such a free hemolysin is present in the spleen it should be demonstrable in extracts of the spleen, and during the past few years several investigators have therefore tested the influence of such extracts upon red cells. The methods employed, based on the technique of Korschun and Morgenroth (18), are similar, but the results obtained have been contradictory.

Korschun and Morgenroth found in several organs a hemolytic substance of unknown origin, coctostabile and soluble in alcohol, which did not arise from constituents of the blood serum and was in no way peculiar to the spleen. Nolf (19), on the other hand, found that the hemolytic power of splenic extract was distinctly greater than that of the liver, mesenteric lymph nodes, or kidneys, but only slightly more than that of the lung. This hemolytic substance was specific for the species and was destroyed at 100° C. Achard, Foix, and Salin (20), repeating these experiments, showed that the final solution was strongly acid, presumably as the result of bacterial action, and that control tests made with precaution as to asepsis were uniformly negative. Widal, Abrami, and Brulé (21) in similar experiments could get no hemolysis with fresh extracts used on the day they were prepared; sometimes, also, extracts twenty-four to forty-eight hours old were without effect. From these results they conclude that the hemolytic substance is not a true hemolysin, but the product of cell autolysis. Iscovesco and Zacchiri (22) had previously shown that after placing the mixture of pulp and saline solution in the thermostat for fifteen to twenty hours, the filtered extract, on the addition of red cells and after standing two and one half hours in the thermostat, showed 2.5 to 8 per cent. hemolysis, as determined by the Dubosc colorimeter, and they conclude that the hemolytic power of splenic extracts is unimportant. Weill (23) found a weakly hemolytic substance in extract of spleen that was inactivated at 56° C. and reactivated with guinea pig serum. This was more powerful than a lymph node extract prepared in the same way, but much less powerful than the extract obtained from the spleen by long maceration. The latter was not destroyed below 80° C., and its action was hindered by adding

fresh serum. Extracts from lymph nodes prepared in the same way showed only slight hemolytic action and those from other organs were negative. Banti (12) and Furno (13) state that fresh extracts of the normal spleen sometimes have no hemolytic action, and sometimes a weak action which is increased on standing twenty-four to forty-eight hours on ice and is not destroyed by heating to 60° or even to 100° C. They consider it a cytohemolysin, normally present in the spleen in small amounts and much increased after the administration of hemolytic agents. Thus we find that Nolf, Weill, Banti, and Furno find splenic extracts to have a hemolytic action greater than that of other organs. Achard, Foix, and Salin, and Widal, Abrami, and Brulé, on the other hand, fail to find any hemolytic action of the fresh extract, and think it is found only after autolysis or bacterial decomposition of the spleen.

Our experiments were made with extracts from the spleens of three dogs. The technique described by Nolf was followed in the main with several additions in the way of control experiments. On washing through the aorta, it was found that the technique which will give a blood-free kidney or liver will not render the spleen bloodless. Various expedients were tried, therefore, to secure a hemoglobin-free extract. It was found that if the spleen, after washing through the aorta, was cut in small pieces and pounded with a pestle against a wire-meshed sieve placed in a mortar, with the aid of frequent washings with salt solution, a blood-free white mass was obtained consisting partly of reticulum and partly of adherent splenic pulp (extract A, table I). As it was possible that the hemolytic substance might not be retained, or in only small amounts, in this fraction, extracts were also made from that part of the spleen that was mashed through the sieve. This residue was of course distinctly blood-tinged, so that it was necessary, in order to remove the blood, to mix it with distilled water, centrifuge, discard the supernatant fluid, and repeat the process until colorless tissues were obtained (extract B, table I). In each case the material thus obtained was mixed with double the amount of salt solution and placed in the refrigerator. Tests were always made with extracts one or two hours old, a small portion being filtered off for this purpose, and in two instances also after eighteen and twenty-four hours. Control tests were made in one experiment with extracts of liver and mesenteric lymph nodes. As it was possible to wash the latter free of blood before removal from the body, an extract was easily obtained by grinding the tissue in sand with mortar

TABLE I.

Character of extract.	Amount of splenic extract in c.c.								Salt solution control.	Distilled water control.
	1.95	1.5	1.0	0.5	0.3	0.2	0.1	0.05		
1. Dog 1. Fresh spleen extract A......	—	V.S.	o	o	o	o	o	o	o	C.
2. Dog 2. Fresh spleen extract A......	—	o	o	o	o	?	o	o	o	C.
3. Same. Extract B........	—	o	o	o	o	o	o	o	o	C.
4. Same. After extraction in ice chest for 24 hrs......	—	V.S.	V.S.	o	o	o	o	o	o	C.
5. Dog 3. Fresh spleen extract A......	V.S.	o	o	o	—	o	o	o	o	C.
6. Spleen extract A after extraction in ice chest for 24 hrs.................	—	o	o	o	o	o	o	o	o	C.
7. Fresh spleen extract (boiled)...............	o	o	o	o	—	o	o	o	o	C.
8. Fresh liver extract........	o	o	o	V.S.	—	o	o	o	o	C.
9. Fresh mesenteric lymph node extract...........	o	o	o	o	—	V.S.	o	o	o	C.
10. Mesenteric lymph node extract after extraction in ice chest for 24 hrs......	—	o	o	?	V.S.	M.	V.S.	o	o	C.

o = no hemolysis; ? = doubtful hemolysis; V.S. = very slight hemolysis; M. = marked hemolysis; C. = complete hemolysis; — = no test.

and pestle and placing it as before in the ice chest with double the amount of salt solution. In two experiments the tests were made on the corpuscles of the animal furnishing the spleen; in one the corpuscles of another dog were used without a difference in result. The preparation of the washed red blood corpuscles, the dilutions, incubation, and so forth, were made according to Nolf's technique. Each tube contained 0.1 of a cubic centimeter of washed dog's corpuscles with varying amounts of splenic extract made up to two cubic centimeters with normal salt solution. Controls were made with normal salt solution and distilled water. The results are presented in table I.

Conclusions.—Fresh extracts of spleen are devoid of definite hemolytic action. Occasional trivial and irregular results, not to be explained, are found, but these occur likewise in the control extracts of liver and mesenteric lymph nodes. Extracts twenty-four hours old, prepared at low temperature, show little or no increase in hemolytic activity. Boiled splenic tissue, extracted in the cold for twenty-four hours, is inert.

THE INFLUENCE OF THE INTRAPERITONEAL INJECTION OF SPLENIC EXTRACT.

The changes in the blood picture following experimental removal of the normal spleen suggest that changes of interest might be produced by a converse procedure; namely, introduction into the body of the products of splenic activity in the form of splenic extract. If temporary anemia follows removal of the spleen, one might expect that some temporary rise in the red cell count might follow the injection of splenic extract.

The literature concerning the spleen contains very few reports on this subject. Danilewsky (24) found a surprising increase in hemoglobin and red blood cells after a single subcutaneous or intraperitoneal injection of extract of spleen. This increase reached its height in from three to seven days and continued as long as the experiment lasted, usually eight days. In dogs with a dietary anemia, splenic extract caused an even greater rise; for example, of 40 per cent. hemoglobin and almost 2,000,000 red cells. Danilewsky assumed that his results were due to a stimulation of the bone marrow. This influence of the splenic extract was not destroyed by heating.

Danilewsky's work, however, is uncontrolled by injection of other organ extracts, and the rise noted extended over a surprisingly long period of time. Silvestri (25) records a single observation in which a dog, presumably dying from anemia, was apparently saved by the injection of splenic extract. In this connection it must also be noted that the clinical literature of this subject contains several reports (26) of the use, with good results, of extracts of spleen and bone marrow in the treatment of anemia.

Method.—We have tested the effect of splenic extract on four dogs, using as controls extracts of other organs similarly prepared and extracts of erythrocytes.

The usual examinations of the blood were made, and also determinations of the resistance of the erythrocytes to hypotonic salt solution and the percentage of skeined cells. As a rule two counts were made before injection, and daily counts after the injection until the blood picture had returned to normal, usually a period of from three to four days. Extracts were prepared from organs removed aseptically from dogs bled to death under ether anesthesia. The finely chopped organ was ground in a sterile mortar to a homogeneous pulp and extracted with double the volume of salt solution for two hours in the ice chest. Ten cubic centimeters of the filtered extract were injected intraperitoneally into dogs of about the same

weight. Defibrinated blood diluted 1 to 20 with normal salt solution was used in ten cubic centimeter amounts to control the possibility of the rise in red cell count being due to the influence of some constituent of the red cells. In no case did peritonitis or other infection result. The result of one of these experiments is shown in table II.

TABLE II.

Date (1914).	Hemoglobin.	Red blood cells.
Feb. 6	102	5,250,000
Feb. 7	101	5,650,000
	(10 c.c. splenic extract No. 16 injected.)	
Feb. 8	110	6,500,000
	(15 c.c. of same extract injected.)	
Feb. 9	110	7,040,000
Feb. 10	105	6,800,000
Feb. 11	96	5,330,000
Feb. 12	95	5,290,000
	(15 c.c. splenic extract No. 88 injected.)	
Feb. 13	101	5,700,000
	(10 c.c. of same extract injected.)	
Feb. 14	104	6,880,000
Feb. 15	98	5,860,000
Feb. 16	96	5,120,000
Feb. 19	106	5,540,000

This experiment shows that intraperitoneal injection of splenic extract causes a sharp rise in hemoglobin and red cell count, lasting only one or two days. This rise is repeated on reinjection of either the same or another splenic extract.

In each of three other experiments with splenic extract an increase in the number of red cells was obtained, but this increase was not always as marked as in the experiment presented; it was nevertheless always greater than that caused by the use of control extracts of liver, kidneys, or blood.

The study of the resistance of the red cells may be dismissed with the statement that no noteworthy differences were found after injection of any extract. The skeined cells also showed no constant change. We had hoped that as the latter are supposed to be young forms of erythrocytes, they would be found to be increased after the injection of splenic extract had caused a rise in the red cell

count. Only once, however, when the percentage rose from 0.5 to 2 per cent., was this noticed. On the other hand, in two experiments they were not found at all in the blood after injection.

Intraperitoneal injection of splenic extract is usually followed by an increase in the total number of leucocytes, consisting chiefly of the polymorphonuclear forms. A similar rise occurred in one of three injections of liver and kidney, and in one of two of defibrinated blood. Several grades of transitional cells appeared in increased numbers. Eosinophils were present in increased numbers in two of the four dogs receiving splenic extracts, but were also definitely increased in two of the five controls.

Conclusions.—Intraperitoneal injection of saline extracts of fresh spleen constantly causes a sharp increase in red cell count and hemoglobin content. The rise is evanescent, lasting but one or two days, and may be followed by an equally evanescent drop below normal. Similarly prepared extracts from other organs fail to give this rise. No noteworthy change is found in the resistance of the red blood cells to hypotonic salt solutions, or in the number of skeined or reticulated erythrocytes, after the injections of the various organ extracts.

A temporary increase of polymorphonuclear and transitional leucocytes usually follows the use of spleen extract, but may occur also, though less frequently, after the injection of liver and kidney.

The constant increase of red cells in the peripheral circulation after injection of spleen, in view of the tendency to anemia following splenectomy, suggests that the spleen normally may exert a stimulating effect upon the formation of red cells in the bone marrow.

THE INFLUENCE OF FEEDING SPLEEN TO SPLENECTOMIZED DOGS.

This study complements that just described in that spleen in large amounts was fed to splenectomized animals. The object was to determine whether through the influence of a possible internal secretion of the spleen the anemia following splenectomy might be prevented. The procedure is of course analogous to thyroid feeding in insufficiency of the thyroid gland, and has an advantage over the injection of extracts in that it may be continued over long periods of

time without the possibility of the complications occasionally occurring after injection. These experiments, it was hoped, would show whether or not the spleen exerts some effect upon the hemopoietic system through peculiar bodies concerned perhaps in an internal secretion. Thus, if the anemia following splenectomy depends upon the absence of a normal stimulus to the hemopoietic system in general, or to some part of it, as the bone marrow, furnished normally by the spleen, the feeding of normal fresh spleen unmodified by heat or chemicals would supply this secretion and there would be no anemia after the removal of the spleen.

Method.—Five dogs were used. Four of these were given a diet consisting of raw hashed beef spleen, lard, and cracker meal in amounts estimated, according to the weight of each animal, to suit its caloric needs. Of these, three were splenectomized and one served as a control. As an added control, a splenectomized dog received a diet in which casein was substituted for beef spleen. The red cells and the hemoglobin were estimated several times before splenectomy and afterwards counted twice a week for three weeks. After this they were counted every week for approximately five weeks. No preliminary counts were made until a dog had been on the special diet for at least a week, and splenectomy was not performed until two weeks later.

Results.—Of the three splenectomized dogs receiving spleen in the diet, one showed a very slight decrease in red cells and hemoglobin, but the other two developed the usual anemia of splenectomy. Thus one with an initial red cell count of 6,200,000 showed on the 12th day 4,710,000 red cells with return on the 54th day to 6,040,000. This animal received daily 150 grams of beef spleen. The other dog receiving daily 275 grams of spleen showed a change in red cell content of about the same degree. In the splenectomized dog not fed spleen, the red cells fell from 5,550,000 to 4,210,000 on the 19th day with return to 5,060,000 on the 54th day. In this dog the hemoglobinemia reached its lowest level (65 per cent.) on the 12th day, and remained at about that point until the 26th day. In neither of the other splenectomized animals receiving spleen did the hemoglobin fall below 75 per cent. The normal dog, receiving 150 grams of spleen daily, showed no change in the blood.

It is evident that in two dogs, despite the feeding of spleen, an anemia was produced that ran a course similar to that which we have previously shown to be the rule in splenectomized dogs (27). In view of these definite results, the absence of marked anemia in the third splenectomized dog must be considered as the result of factors other than the feeding of spleen.

Incidentally it was found that the resistance of the red cells to hypotonic salt solution was increased in all splenectomized dogs, thus confirming the work of Karsner and Pearce (28).

Conclusion.—The feeding of fresh raw spleen to splenectomized dogs has no clearly defined influence in preventing the anemia which usually occurs after splenectomy.

GENERAL SUMMARY.

1. The blood of the splenic artery and vein shows either no differences, or only such slight irregular variations as may be due to the errors inherent in hematologic methods, or are common to arterial and venous blood of the general circulation.

2. The observation of Banti and Furno that free hemoglobin occurs in the blood of the splenic vein is not confirmed.

3. Extracts of the spleen have no definite hemolytic action *in vitro.*

4. Intraperitoneal injection of fresh saline extracts of the spleen causes in the dog a sharp increase in the number of red cells and the hemoglobin content which lasts for one or two days and may recur on a second injection. Extracts of liver, kidney, and erythrocytes similarly prepared do not give this effect. This observation supports Danilewsky's theory that the spleen may exert a stimulating effect upon the formation of red cells in the bone marrow.

5. On the other hand, the feeding of raw beef spleen to splenectomized dogs over long periods of time has no clearly defined influence in preventing the anemia which usually follows splenectomy.

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THE RELATION OF THE SPLEEN TO BLOOD DESTRUCTION AND REGENERATION AND TO HEMOLYTIC JAUNDICE.

XI. The Influence of the Spleen on Iron Metabolism.*

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This investigation was undertaken to determine whether the tendency to anemia in splenectomized dogs and the delayed regeneration of the blood after the administration of hemolytic agents to such dogs[1] might be due in part to some influence of the spleen upon the iron metabolism, as has been claimed by Asher (1).

Our present knowledge concerning iron metabolism may be summarized as follows: Iron is absorbed only to a very limited extent from the gastro-intestinal tract, so that when abundant in the food it passes for the most part unchanged from the intestine in the feces. As much as is absorbed is taken up chiefly from the small intestine and carried by the lymph to be deposited in the liver and to a lesser extent in the spleen, bone marrow, and perhaps elsewhere, and this occurs whether the iron be in intimate organic combination, the so called food iron, incapable of giving the characteristic microchemical reaction, or whether it be in the form of an organic or inorganic salt of iron. Moreover, from the work of Häusermann (2) and of Abderhalden (3), it appears that though iron salts are absorbed, the body is unable, or but very poorly able, to utilize them for the building of hemoglobin, being dependent for this constructive work upon the intimately combined food iron. On the other hand, iron salts are effective stimulants to the blood-

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[1] *Jour. Exper. Med.*, 1912, xvi, 363, 375, 758, 769, 780; 1913, xviii, 487, 494, 665; 1914, xx, 19, 108.

forming organs and conspicuously increase the utilization by them of the food iron.

The elimination of iron occurs almost wholly through the intestines, especially the colon, the quantity passing out in the urine constituting less than 1 per cent. of the total excretion in man and the dog. In the fasting dog the output found by von Voit (4) was 0.60 of a milligram per kilo of body-weight per day, and on an adequate, but iron-poor diet, Gottlieb's (5) dog excreted 0.34 of a milligram. For man the figures are lower. Cetti and Breithaupt (6), while fasting, eliminated about 0.10 to 0.13 of a milligram per kilo per day; and in various studies on man 0.10 to 0.25 of a milligram per kilo per day have been found to be the intake required to maintain iron equilibrium. However, there is every reason to believe, as is suggested by the work of Schmidt (7), who fed mice for months on a diet extremely poor in iron, but obtained no fall in the hemoglobin, that the organism possesses great power of conserving its iron and of reutilizing it through some form of intermediary metabolism. When, however, Schmidt withdrew iron from the diet for several generations, the younger generations were extremely anemic and this anemia disappeared upon restoring iron to the diet. As the iron-poor diet led to the disappearance of microchemically demonstrable iron from the liver, but affected to a much slighter degree that of the spleen, Schmidt concluded that the liver is the depot for iron from the food, and that the spleen, on the other hand, is the depot for iron from tissue and erythrocyte catabolism and thus an important factor in the intermediary metabolism of iron.

If the spleen plays this part in iron metabolism, its absence might well interfere with the reutilization of iron by the organism and lead to an increased iron elimination, and this Asher and his co-workers, Grossenbacher and Zimmermann, claim to have demonstrated in dogs. They studied the iron elimination of four puppies from two litters; one from each litter was splenectomized and one from each was kept as a control. The iron estimations were made at intervals of a few weeks, two months, and ten months after splenectomy, and in all their experiments they found an output much higher, often double, in the splenectomized animals as compared with the controls.

Bayer (8) has made some studies on man of the iron elimination following splenectomy for rupture of the spleen or for Banti's disease, and has compared the output for a certain number of days on known diets with that of control cases on the same diets. His results are summarized in the following table.

TABLE I.

Disease.	Age in yrs.	Time after splenectomy.	Output of iron in mg. per kilo per dy.	
			Splenectomized.	Control.
Spleen rupture.........	16	2 wks.	0.22	0.16
			0.15	0.18[2]
Spleen rupture.........	16	3 wks.	0.30	0.22
			0.17	0.08
			0.51	0.50
Banti's disease.........	25	6 mos.	0.19	0.18[2]
Banti's disease.........	19	2 yrs.	0.19	0.18[2]

From these experiments the author concludes that there is an increased output of iron soon after splenectomy, as shown by the second observation in the table, but that later the elimination returns to normal. Bayer's statement that certain of his diets contained 0.24 of a gram of iron per day is probably an error since a diet of the general character that he describes would certainly have a much lower iron content.

METHODS.

In our earlier experiments we studied the iron elimination during four-day periods, but found that these periods led to irregular results. In the work here reported, therefore, we present only observations based on periods of nine or ten days' duration.

The animals were placed in metabolism cages with glass floors and after they had been fed for several days on constant weighed amounts of the diet selected, the rectum was emptied by the use of morphin; iron-free charcoal was added to the next feeding, and the collection of feces was begun from the appearance of the charcoal; at the close of the period the rectum was again emptied with morphin, carmine was added to the next feeding, and the feces were collected until carmine appeared in them. In the earlier experiments

[2] These three figures are merely repetitions of a single control experiment.

the urine also was analyzed, but as only traces of iron, less than 1 per cent. of the total elimination, were found the urine was omitted in our later analyses. To avoid the introduction of extraneous iron, the feces were collected by means of a nickel spatula soon after being passed.

In one group of experiments representing the earlier periods after operation, we have studied the output of iron on the same dogs, both before and after splenectomy, without a change in diet. In another group, representing later periods, we have compared the output of normal control dogs with that of splenectomized dogs of approximately the same weight on corresponding diets.

The analyses were made by the method of Ripper and Schwarzer (9), slightly modified. The feces collected for the entire period are placed in a quartz dish, dried, and ashed dry. The ash is extracted with boiling concentrated hydrochloric acid and filtered, and the residue washed with 20 per cent. hydrochloric acid. The residue and filter paper are re-ashed and the extraction is repeated. This ashing and extraction is continued until the extract ceases to give a positive test with potassium sulphocyanide.

The total filtrate is made up to a known volume and two duplicate portions, containing presumably two to five milligrams of iron, are taken. To each is added one cubic centimeter of hydrogen peroxide (Merck's Blue label), and the solution evaporated to dryness on a water bath. The residue is then redissolved in one cubic centimeter of 20 per cent. hydrochloric acid and twenty cubic centimeters of boiling water are used in four small portions, and then this washing with acid and water is repeated. In the course of the manipulation the entire solution is brought into a 200 cubic centimeter Erlenmeyer flask.

All the specimens to be analyzed at one time having been brought to this stage, a standard is prepared by placing into each of two 200 cubic centimeter Erlenmeyer flasks forty cubic centimeters of a quantitative ferric chloride solution containing about 0.002 of a gram of iron. To each of the flasks, those containing the specimens and the two containing the standard, there are added in rapid succession four grams of potassium iodide; the flasks are then immediately stoppered and placed in a water bath at 60° C. for ten

minutes. At the end of this time the flasks are removed, and to each 100 cubic centimeters of cold water are immediately added and the flask is restoppered.

To each flask in turn is added starch solution, and the contents are titrated with sodium thiosulphate solution, approximately N/250, until disappearance of the blue color, and then they are immediately titrated with weak iodin solution back to the first reappearance of the blue color. In each analysis the thiosulphate solution is freshly prepared and standardized against the two flasks of known ferric chloride solution, and the iodin solution also is freshly prepared and standardized against the thiosulphate solution. The precision of the titration method is found to be greatly enhanced by the titration back with iodin to the first reappearance of the blue color and calculation accordingly of the thiosulphate end point.

In control experiments performed by adding known amounts of iron to one of identical pairs of samples of ash of feces, an error of about 2 per cent. was observed.

The food used in these experiments consisted of casein, cracker meal, lard, and fresh beef heart in proportions designed to give the desired amount of iron. The iron content of the food was determined by analyzing many large portions (each 50 to 400 grams) of the beef heart, cracker, and casein and obtaining average figures for use in calculating the iron content of the diets employed.

RESULTS.

In the accompanying tables are given in detail the final figures obtained in our studies. The experiments are divided into two groups. First, five animals were studied both before and for two weeks after splenectomy, on a constant diet throughout; these are arranged in table II according to the iron content of the diet. Second, a group of six animals (table III), three normal controls and three splenectomized animals, were studied at longer periods after splenectomy; these were of about the same weight and were on diets of the same general character, but varying in the content of iron.

Inspection of table II shows that the iron output of dogs 88 and

35 is unchanged by splenectomy, but that dogs 30, 44, and 79 show some increase. On the other hand, in table III, it will be seen that all three splenectomized dogs exhibit an output of iron as compared with the intake closely comparable with that of the controls. From our studies it would appear therefore that during the first two weeks after splenectomy, some, but not all dogs show a slight increase in the output of iron, but that at 1 month, 9 months, and 20 months after splenectomy we find no indication of an increased iron output. The occasional evanescent and inconstant increase in elimination of iron does not justify the conclusion that the spleen exerts an important influence on iron metabolism. Our

TABLE II.

Dog No.	Average weight.	Duration of periods.	Intake.[3]	Output.[3]		Time after splenectomy.
				Before splenectomy.	After splenectomy.	
88	7,000	10 dys.	0.27	0.67	0.70	4–14 dys.
30	5,340	9 dys.	0.30	0.36	0.55	1–10 dys.
35	7,720	9 dys.	0.64	0.87	0.81	1–10 dys.
44	9,000	9 dys.	1.57	1.89	2.10	1–10 dys.
79	9,000	9 dys.	1.71	1.88	2.21	6–15 dys.

TABLE III.

Dog No.	Controls.				Time after splenectomy.
	Average weight.	Duration of period.	Intake.[4]	Output.[4]	
79	9,000	9 dys.	1.00	1.42	
44	9,000	9 dys.	1.57	1.89	
79	9,000	9 dys.	1.71	1.88	
	Splenectomized.				
83	8,400	10 dys.	1.42	1.39	27–37 dys.
9	8,800	9 dys.	1.35	1.56	9 mos.
51	10,000	9 dys.	1.32	1.42	20 mos.

results are obviously different from those of Asher and his associates, and as a possible explanation of this we would call attention to the extreme shortness of the periods—one to three days—employed

[3] Figures expressing intake and output indicate milligrams of iron per kilo per day.

[4] Figures expressing intake and output indicate milligrams of iron per kilo per day.

by Asher and Grossenbacher, and to their failure to mark in any way the stools. In the studies of output ten months after splenectomy, as given by Asher and Zimmermann, the splenectomized dog in most of the experiments was much larger than the control, so that if the iron output of their dogs be calculated per kilo of body-weight it will be found that the output of the splenectomized animals approaches very closely that of the normal controls, and is in some instances identical. It seems possible that in these studies ten months after splenectomy the increased iron output of the splenectomized animals was due rather to the size of the animal than to the splenectomy, and it is doubtful, therefore, whether the conclusions of Asher and Zimmermann, based on these experiments, are justified.

CONCLUSIONS.

Our studies give evidence of increase in the iron elimination in three of five dogs during a period of two weeks following splenectomy, but not in two other dogs. The occasional increased output of iron may have some relation to the anemia which occurs in the early weeks after splenectomy and which varies in degree in different animals.

No evidence was secured of an increase in the iron output at 1, 9, and 20 months after splenectomy.

From our own studies and from examination of the literature of the subject, we conclude that the spleen does not exercise a constant and important influence upon the iron metabolism of the body.

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THE EFFECT OF THE VARIATION IN THE OSMOTIC TENSION AND OF THE DILUTION OF CULTURE MEDIA ON THE CELL PROLIFERATION OF CONNECTIVE TISSUE.*

By ALBERT H. EBELING.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

Plates 11 to 14.

In his fundamental experiments Jacques Loeb has shown the importance of the osmotic tension of water for the growth of marine organisms. Carrel and Burrows,[1] by cultivating for a few days embryonic chick spleen in diluted plasma and in plasma the osmotic tension of which had been modified, attempted to determine what factors could activate the rate of growth of the tissues of higher animals. These experiments were made before Carrel developed the technique which rendered possible the permanent life of connective tissue *in vitro*. Afterwards it became apparent that the modifications of the medium acted differently on tissues proliferating for many generations and tissues maintained for a few days in a condition of survival, and that the conclusions in the article of Carrel and Burrows needed to be modified.

Dr. Carrel gained the impression that it would be necessary, in order to know the influences on cell proliferation of the variation in the osmotic tension and of the dilution of the culture media, to observe for many passages the growth of a tissue in a specific medium. The present experiments were therefore undertaken to determine definitely to what extent strains of connective tissue, kept in a condition of active life, *in vitro,* for many generations, were influenced by the modification of the medium. During the time this work was in progress, an article by Lambert[2] was published on the effect of dilution of plasma on the growth of cells in tissue

* Received for publication, May 25, 1914.

[1] Carrel, A., and Burrows, M. T., *Jour. Exper. Med.*, 1911, xiii, 562.

[2] Lambert, R. A., *Jour. Exper. Med.*, 1914, xix, 398.

cultures. It was stated that plasma with isotonic solutions causes a more extensive migration in cultures of cells of the actively migratory type, such as those of spleen and bone marrow. Dilution with a limited quantity of distilled water produced the same effect. Less actively motile cells are influenced little or not at all by dilution; also, dilution of the plasma with either isotonic solutions or distilled water is without effect on the cell multiplication. His experiments did not differ in method from those of Carrel and Burrows and are, therefore, open to the same objection.

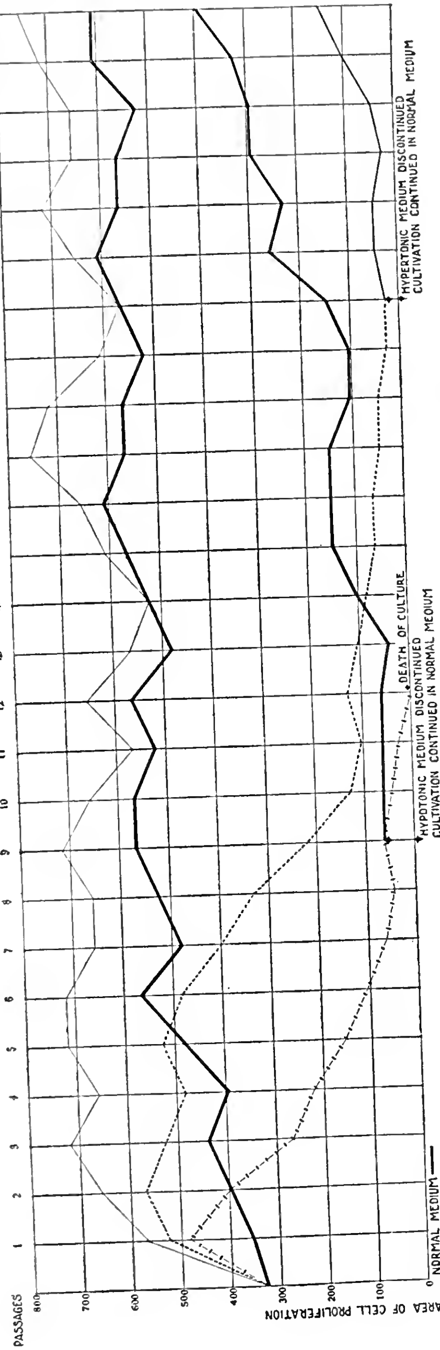
TECHNIQUE.

The strain of connective tissue used as a control was cultivated in a medium, designated normal, composed of one part of freshly prepared embryonic chick extract and of two parts of normal chicken plasma.

Hypotonic medium was prepared by adding two parts of distilled water to three parts of normal plasma and one part of fresh embryonic chick extract.

Preliminary experiments were made to determine the concentration of the salt solution which was added to normal plasma in order to render it sufficiently hypertonic. It was found that a 2.5 per cent. sodium chloride solution was too concentrated. When employed in a culture medium it proved distinctly unfavorable to cell proliferation after one or two passages. A medium containing a 2 per cent. solution of sodium chloride in the same proportion showed that it was possible to keep strains of connective tissue cells in a condition of active life for a number of passages. 1 and 1.5 per cent. solutions were also tried, but the hypertonicity produced thereby was found to be so slight as to approach the conditions which prevailed when normal plasma was diluted with Ringer solution. The hypertonic medium was prepared by adding two parts of a 2 per cent. solution of sodium chloride to three parts of normal chicken plasma and one part of fresh embryonic chick extract.

Diluted medium was prepared by adding two parts of Ringer solution to three parts of normal chicken plasma and one part of fresh embryonic chick extract. Thus, the three modified media, hypotonic, hypertonic, and diluted, contained the same relative amount of normal plasma and fresh embryonic chick extract.



TEXT-FIG. 1. The average area of cell proliferation which developed during forty-eight-hour intervals (one passage) of incubation at 40° C. in cultures of connective tissue which were cultivated in normal, hypertonic, hypotonic, and diluted media, respectively.

Two strains of connective tissue cells were used in these experiments. One strain was derived from a fragment of heart tissue from a chick embryo seven days old, extirpated on January 17, 1912; the other from a fragment of heart tissue from a chick embryo eight days old, isolated on February 18, 1913.

Cultures were made by subdividing fragments of these two strains of connective tissue. The pieces were washed in Ringer solution for three quarters of a minute to one minute and cultivated respectively in normal, hypotonic, hypertonic, and diluted medium. After forty-eight hours' incubation the cultures were washed in Ringer solution as before, transferred into fresh medium, and again incubated. After incubation for one, six, twenty-four, and forty-eight hours, observations were made, and in some instances cultures were fixed and stained. To determine the influence of modified media on the proliferation of connective tissue cells, the growth obtained in a culture, after stated periods in normal medium, was taken as a standard of comparison. The extent of this growth was measured with an ocular micrometer and recorded. The accompanying chart shows, in curves, the relative area of cell proliferation which developed during a passage (forty-eight hours) in cultures which had been grown in modified media. Calculations were made by measuring in the different media the width of the zone of new growth which had developed during a passage. The measurements for all cultures which had been cultivated in the same medium were averaged and the area was computed. The relative density of cell proliferation was determined by observations on stained preparations, and photomicrographs of the preparations were made.

EXPERIMENTS.

Continued cultivation of connective tissue in hypertonic medium produced the following results: When a culture was transferred to hypertonic medium from a normal medium in which the width of the area of cell proliferation after forty-eight hours' incubation was equal to seven divisions of the ocular micrometer, observations showed, after one hour's incubation, no evidence of cell proliferation; after six hours' incubation a few elongated cells were observed spreading from the periphery of the central fragment into the

medium; after twenty-four hours the area of cell proliferation measured, on the average, six and a half divisions, slightly more than the area of cell proliferation in the control, which measured six divisions. Stained preparations at this stage of incubation showed the area to be denser than the control. After forty-eight hours' incubation the zone of proliferating cells measured nine divisions, and in the control seven and a half divisions. The density was greater in comparison with the normal control and the contrast could be observed in the living culture. Examination of the cells in these cultures showed the presence of many refractile globules of varying size in the cytoplasm of the cell. In the control, cells showed small refractile globules, few in number and fairly uniform in size. Preparations stained with Sudan III and hematoxylin showed these refractile globules to be fatty substances which had accumulated in the cell cytoplasm. A third passage into hypertonic medium after forty-eight hours' incubation produced a less extensive, though quite as dense an area of cell proliferation as the control. The zone measured six divisions, and the control nine and a half. The amount of fat present in the cytoplasm of the cell was observed to be apparently as great as in the previous passage. There was no increased amount of fat present in the control over that present in the new cells which developed in the previous passage. A fourth and fifth passage, after forty-eight hours' incubation, respectively, showed a decided decrease in extent of new growth. The area of cell proliferation appeared to be as dense as the area of cell proliferation in the control, but the central portion of the culture had become a thick and opaque mass. The cells were observed to be filled with large fat granules. After the sixth and seventh passages the extent of cell proliferation was decidedly less than the control, measuring two and a half divisions, whereas the extent of growth in the control measured nine and a half. The central portion of the cultures had contracted into a very dense, spherical, opaque mass. Further passages into hypertonic medium resulted in a rapid degeneration of the culture, with central necrosis and death after the eleventh or twelfth passage. In some instances tissues which had been cultivated for nine passages (eighteen days) in hypertonic medium were revived by again cultivating them in

normal medium, and after seventeen passages (thirty-four days) the extent of cell proliferation was approaching normal. In the first few days during which a culture was cultivated in hypertonic medium the area of new growth was more extensive and apparently more dense than in the control. After that time this area decreased rapidly and eventually the culture died.

Cultivation of connective tissue in hypotonic plasma after one hour's incubation showed new cells budding out from the peripheral portions of the central fragment. The same condition was observed in the control. After six hours' incubation the area of cell proliferation was about the same as the control, but in twenty-four hours the zone of proliferating cells was more extensive than in the control, measuring about eight and a half divisions in width, whereas the width of the area in the control measured slightly over seven divisions. After forty-eight hours' incubation the increase in extent of cell proliferation was decidedly greater than the control, measuring about nine and a half divisions, as compared with seven and a half divisions in the control. The density of growth, however, was less than the control. Stained preparations showed fewer planes in which cell proliferation had taken place. After the second and third passages, respectively, the extent of growth was still observed to be greater (about nine and a half divisions) than the control (about seven and a half divisions). The accumulation of fat globules in the cytoplasm of the cells was practically the same as the control. After the fourth and fifth passages the area of proliferating cells was slightly greater than the control, but the relative density was less in the former. After the sixth and seventh passages the extent of cell proliferation decreased and was observed to be less than the control. The cells showed an increase in fat accumulation over the control and the cells appeared loosely joined, forming a large meshed network of interlacing cells. Continued cultivation in hypotonic medium up to twenty passages (forty days) showed a gradual decrease in the extent of growth. The central fragment became dense and opaque and large fat globules were observed in the cytoplasm of the cells. After twenty passages in hypotonic medium some cultures were again cultivated in normal medium. They usually recovered after five or six passages. During a period last-

ing about ten days, a culture cultivated in hypotonic medium showed that the area of cell proliferation was more extensive though less dense than the normal control. After that time the extent of growth decreased gradually and finally cell proliferation became sluggish. It was possible to revive again a culture at this stage.

The cultivation of cultures of connective tissue in diluted medium after one hour's incubation showed a number of new cells growing out into the medium from the periphery of the central fragment. In six hours an appreciable crown of new cells encircled the central portion of tissue, and after twenty-four hours the area of new growth was more extensive (about eight and a half divisions in width) than the control (about seven divisions in width). After forty-eight hours it was observed that the area of cell proliferation which had developed was decidedly more extensive (width of area eleven divisions) than the control (width of area seven and a half). Stained preparations showed this area to be less dense than the control, but there was no increase in the amount of fat globules present in the cytoplasm of the cells. A second, third, and fourth passage showed an increase in area over the control and many passages thereafter (twenty-two passages, forty-four days) showed that this increase in area of cell proliferation over the normal was fairly constant. This point is shown in the text-figure 1. The fluctuations of growth which were observed in the control were also observed at the same time in the experiment. When the extent of cell proliferation was less extensive in normal medium than in the previous passage, it was found that there was a relative decrease, on most occasions, in the extent of cell proliferation in diluted medium. The stained preparations of cultures cultivated in diluted medium for many passages showed an extensive, loosely meshed network of elongated cells. The control showed a more densely packed mass of elongated cells, but the area of cell proliferation was less. Figure 1 represents an entire culture of connective tissue forty-eight hours after passage into normal medium, which had been cultivated for about two hundred passages in this medium. Figure 2 shows an entire culture of connective tissue forty-eight hours after the last passage into diluted medium. The last passage was the twenty-fifth consecutive passage into diluted medium. Figure

3 is a higher magnification of part of a control culture after forty-eight hours' incubation. Figure 4 shows a portion of the area of cell proliferation which developed in a culture in forty-eight hours in diluted medium, after twenty-two passages into the same medium. The relative increase in the extent of cell proliferation in diluted medium, over the control, remained fairly constant through the total number of passages, although no actual increase in mass was observed (text-figure 1).

When connective tissue was cultivated in hypertonic medium for one passage and then in hypotonic medium, observations showed no marked differences in cell proliferation, but alternate passages from hypertonic to hypotonic medium for three or four passages caused death of the culture. Alternate passages from hypertonic medium into diluted medium proved unfavorable after three or four passages, and usually resulted in death of the culture after five generations. Alternate passages from hypertonic medium into normal medium proved unfavorable after five passages, and when continued for ten passages the culture died. Alternate passages from hypotonic into normal medium were continued for ten generations. A gradual decrease in the extent and activity of cell proliferation was observed and this treatment proved unfavorable for growth in the culture. Observations showed that alternate passages from normal medium into diluted medium did not appear to affect the dynamic condition of the culture, although, relatively, there appeared to be a denser area of cell proliferation in normal medium and a less dense but more extensive area of growth in diluted medium. Alternate passages of connective tissue into modified media in most instances produced unfavorable results.

SUMMARY.

For the first few days of cultivation of connective tissue in hypertonic, hypotonic, and diluted medium, cell proliferation was stimulated. The first outgrowths of new cells in the modified media did not occur sooner than in normal medium. In hypertonic medium the density of the area of cell proliferation appeared to be greater than the control, but in hypotonic or diluted medium there seemed to be no increase in actual mass over the control. These observa-

tions confirm the conclusions of Carrel and Burrows, as well as those of Lambert.

Subsequent to the first few days of cultivation in hypertonic medium the area of cell proliferation decreased and in a short time conditions developed which were unfavorable to growth, and finally resulted in death of the culture, unless it revived before this stage. Hypotonic medium after about ten days no longer caused more extensive areas of proliferating cells; but instead, the extent of new growth gradually decreased, and the culture merely remained alive unless revived. In diluted medium the extent of the area of cell proliferation remained greater with no actual increase in mass. The area of cell proliferation which is observed during the first few days in a culture of fresh tissue recently extirpated does not indicate the actual influence of modified media. It was only after continued cultivation of strains of connective tissue in these modified media that their influence on cell proliferation was determined.

CONCLUSION.

An increase or decrease in the osmotic tension of the culture medium at first stimulates cell proliferation, but eventually retards it and proves to be unfavorable to growth. Dilution of the medium without change of the osmotic tension produces a more extensive zone of cell proliferation but no increase in the actual mass of newly formed tissue. A culture of connective tissue, which has been growing under unfavorable conditions, due to changing the osmotic tension of the medium in which it has been cultivated, is capable of being revived.

EXPLANATION OF PLATES.

Plate 11.

Fig. 1. The extent of growth obtained after forty-eight hours' incubation in normal medium. This culture had been cultivated in normal medium for about two hundred passages. Control for culture in figure 2. Stained preparation; low power.

Plate 12.

Fig. 2. The extent of growth obtained after forty-eight hours' incubation in diluted medium. This culture had been cultivated in diluted medium for twenty-five passages (fifty days). Stained preparation; same magnification as figure 1.

PLATE 13.

FIG. 3. The extent of the area of cell proliferation which developed after forty-eight hours' incubation in a normal control. Stained preparation; higher power.

PLATE 14.

FIG. 4. The area of cell proliferation which developed after forty-eight hours' incubation in diluted plasma. This culture had been cultivated in the same medium for twenty-three generations (forty-six days). Stained preparation; same magnification as figure 3.

THE CULTIVATION OF HUMAN SARCOMATOUS TISSUE IN VITRO.*

By JOSEPH R. LOSEE, M.D., and ALBERT H. EBELING.

(From the Laboratories of the New York Lying-In Hospital and of The Rockefeller Institute for Medical Research.)

Plates 15 to 17.

The first attempt to cultivate human malignant tumor *in vitro* was made in 1911 by Carrel and Burrows.[1] Small fragments of tumor were cultivated in normal human plasma and incubated. It was observed in some cases that after a few days the fragments were surrounded by many cells; but generally liquefaction of the medium occurred. The tissues were kept in a condition of survival for a few days, but no real cultures were obtained.

Lately it became possible to keep human fetal tissue, derived from fresh cadavers, in a condition of independent life for several generations,[2] and we therefore attempted to cultivate human sarcomatous tissue in the same manner.

TECHNIQUE.

The medium employed in these experiments was composed of equal parts of normal human plasma and Ringer solution and varying quantities of extract.

The extract was prepared by cutting tissues obtained from fresh fetal cadavers into small pieces, and adding an equal quantity of Ringer solution. After forty-eight hours in cold storage the substance was centrifuged and the supernatant fluid pipetted off. This fluid was used as extract in the making of cultures.

The tissues employed were obtained from recently excised sarcomatous growths,[3] and cultures were made about one and a half hours

* Received for publication, June 3, 1914.

[1] Carrel, A., and Burrows, M. T., *Jour. Exper. Med.*, 1911, xiii, 387.

[2] Losee, J. R., and Ebeling, A. H., *Jour. Exper. Med.*, 1914, xix, 593.

[3] The sarcomatous growths were obtained by Dr. Carrel, through the courtesy of Dr. W. B. Coley, from some of his cases at the General Memorial Hospital, New York. Immediately after excision the tissues were carried to the Laboratories of the New York Lying-In Hospital.

after excision. The primitive cultures were made by putting small, thin fragments of this tissue into the medium. After coagulation the cultures were immediately placed in the incubator and incubated at 38° C. for 24, 48, and 72 hours, the time of passage into fresh medium being governed by conditions which developed in the culture. Before the fragments in cultures were transferred into fresh medium they were washed in Ringer solution for about one minute.

EXPERIMENTS.

Experiment 1, Series 1, Cultures 1, 2, 3, and 4.—Fragments from the periphery of an osteosarcoma[4] were cultivated in equal parts of human plasma and Ringer solution, to which one fourth part of extract was added. The cultures were made about one hour and a half after excision of the growth and incubated.

After 1, 6, and 18 hours' incubation there was no evidence of cell proliferation in any of the cultures. In twenty-four hours there was still no growth to be observed and the medium had become liquefied around the fragments. The cultures were therefore washed and changed into fresh medium (first passage), to which one half part of extract was added. After twenty-four hours' incubation, growth was found to be present in culture 3, but no growth was observed in cultures 1, 2, and 4, and in forty-eight hours there was still no evidence of cell proliferation in these cultures; the medium had become liquefied, and they were therefore discarded.

Culture 3 was cultivated in the same medium (second passage), and in forty-eight hours growth was apparent. This culture was transferred into fresh medium for twelve passages, during which time (twenty-one days) growth was observed after each transfer into fresh medium. The culture was stained and photographed (figure 1).

Series 2, Cultures 1, 2, 3, and 4.—Fragments from the peripheral area of the same growth were cultivated in the same manner as series 1.

After 1, 6, and 18 hours' incubation there was no evidence of cell proliferation. The medium was still in good condition. In twenty-four hours no growth was observed, and the medium about the

[4] Pathological diagnosis: large round cell sarcoma.

pieces in cultures 1, 2, and 3 had become liquefied. Culture 4 was allowed to remain in the incubator. It was examined after forty-eight and seventy-two hours, but there was no evidence of cell proliferation, although the medium was still in good condition. The culture was discarded. After twenty-four hours cultures 1, 2, and 3 were changed into fresh medium (first passage), to which one half part of extract was added.

Culture 1 after twenty-four hours showed no growth, and the medium was completely liquefied. It was changed into fresh medium (second passage), the same proportion of extract being added as in the previous passage. In twenty-four hours a few scattered cells were observed, but after forty-eight and seventy-two hours there was no further increase in the extent of cell proliferation. The culture was discarded.

Culture 2 (first passage) after twenty-four hours' incubation showed an area of cell proliferation, with no liquefaction of the medium. In seventy-two hours a good growth had developed. The medium was slightly liquefied. It was then changed into fresh medium (second passage), to which one fourth part of extract was added. After twenty-four hours growth had developed, and in forty-eight hours the area of cell proliferation was more extensive, but liquefaction of the medium had developed. The culture was changed into fresh medium (third passage) with the same proportion of extract added as in the previous passage. After twenty-four hours a few cells were observed in the medium surrounding the central fragment. In forty-eight hours there was no increase in the extent of growth and the medium had liquefied. The culture was again transferred into fresh medium (fourth passage), one part of extract being added. In twenty-four hours the medium had become liquefied and no growth was observed. The culture was changed (fifth passage) into fresh medium, the same proportion of extract being added as in the previous passage. In twenty-four hours growth had developed, and in forty-eight hours it was more extensive, but the medium was almost completely liquefied. The culture was transferred (sixth passage) into fresh medium in the same manner as in the previous passage. After twenty-four hours cell proliferation was observed, but small colonies of bacteria had also developed. The infection was general and the culture was discarded.

Culture 3 (first passage), after twenty-four hours' incubation, showed a few scattered cells which had grown out from the original fragment. In forty-eight hours the growth of new cells had increased, but the medium was slightly liquefied. The culture was then treated (second passage) in the same manner as in the previous passage. After twenty-four hours good growth had developed and the medium was in good condition. After forty-eight hours the growth was more extensive, but after seventy-two hours there was no further increase and the medium was slightly liquefied. The third passage into fresh medium was then made, the proportion of extract being increased to one part. After twenty-four hours' incubation the growth had developed and the medium was in good condition. In forty-eight hours the culture was growing actively and the medium had become slightly liquefied. The culture was subsequently changed into fresh medium for twenty-one more passages. The extent of growth fluctuated and gradually decreased. In the fifteenth passage the culture was divided and two cultures were made; one of the cultures (figure 2) was fixed after forty-eight hours' incubation. After the twenty-fourth passage cell proliferation stopped. This culture was transferred twenty-five times, during a period of fifty-one days. The entire history is given in table I, which shows the passage, treatment, and observations that were made on culture 3.

Experiment 2.—A series of cultures, Nos. 1, 2, 3, and 4, was made from fragments of the periphery of a large round cell sarcoma,[5] and cultivated in equal parts of plasma and Ringer solution. After twenty-four hours there was no evidence of growth in any of the cultures and the medium was in good condition. In forty-eight hours there was evidence of cell proliferation in all cultures. After seventy-two hours the area of cell proliferation had increased, but the medium in cultures 3 and 4 had become liquefied. Cultures 1 and 2 were stained.

Cultures 3 and 4 were changed into fresh medium (first passage), and one part of extract was added to the medium. Both cultures developed good growth in forty-eight hours with no liquefaction of the medium. They were again transferred into fresh medium

[5] Pathological diagnosis: large round cell sarcoma.

TABLE I.

Passage.	Date (1913).	Treatment of culture.	Observations.
Experiment 206-3	Nov. 18	Culture of a fragment of the peripheral part of an osteosarcoma, cultivated in 1 part of human plasma, 1 part of Ringer solution, and ¼ part of extract	Nov. 19. Medium liquefied; no growth.
1	Nov. 19	Washed in Ringer solution for 1 minute, cultivated in 1 part of human plasma, 1 part of Ringer solution, and ½ part of extract	Nov. 20. A few scattered cells; medium in good condition. Nov. 21. Good growth; medium slightly liquefied.
2	Nov. 21	Treated in the same manner as in previous passage	Nov. 22. Good growth; medium in good condition. Nov. 23. Growth more extensive; medium in good condition. Nov. 24. No increase in extent of growth; medium slightly liquefied.
3	Nov. 24	Washed in Ringer solution for 1 minute, cultivated in 1 part of human plasma, 1 part of Ringer solution, and 1 part of extract	Nov. 25. Good growth; medium in good condition. Nov. 26. Growing actively; medium slightly liquefied.
4	Nov. 26	Washed in Ringer solution for 1 minute and cultivated in the same medium	Nov. 27. Good growth; medium in good condition. Nov. 28. Growth more extensive; medium in good condition. Nov. 29. No increase in extent of growth; medium liquefied.
5	Nov. 29	Washed in Ringer solution for 1 minute and cultivated in the same medium	Nov. 30. A few cells; medium in good condition. Dec. 1. A few cells; medium slightly liquefied.
6	Dec. 1	Washed in Ringer solution for 1 minute and cultivated in the same medium	Dec. 2. A few scattered cells; medium in good condition. Dec. 3. Good growth; medium in good condition.
7	Dec. 3	Washed in Ringer solution for 1 minute and cultivated in the same medium	Dec. 4. Good growth; medium in good condition. Dec. 5. Very good growth; medium in good condition.
8	Dec. 5	Washed in Ringer solution for 1 minute and cultivated in the same medium	Dec. 6. Good growth; medium slightly liquefied.
9	Dec. 6	Washed in Ringer solution for 1 minute and cultivated in the same medium	Dec. 7. Good growth; medium in good condition. Dec. 8. More extensive growth; medium liquefied.
10	Dec. 8	Washed in Ringer solution for 1 minute and cultivated in the same medium	Dec. 9. Good growth; medium partially liquefied. Dec. 10. No increase in amount of growth; medium liquefied.

TABLE I.—*Continued.*

Passage.	Date (1913).	Treatment of culture.	Observations.
11	Dec. 10	Washed in Ringer solution for 1 minute and cultivated in the same medium	Dec. 11. Good growth; medium in good condition. Dec. 12. Growth increased; medium in good condition.
12	Dec. 12	Washed in Ringer solution for 1 minute and cultivated in the same medium	Dec. 14. Good growth; medium in good condition. Dec. 15. Very good growth; medium in good condition.
13	Dec. 15	Washed in Ringer solution for 1 minute and cultivated in the same medium	Dec. 16. Growing; medium in good condition. Dec. 17. Very good growth; medium slightly liquefied.
14	Dec. 17	Washed in Ringer solution for 1 minute and cultivated in the same medium	Dec. 18. Good growth; medium in good condition. Dec. 19. Extensive growth; medium in good condition.
15	Dec. 19	Washed in Ringer solution for 1 minute, divided into 2 parts, and cultivated in the same medium (cultures 1 and 2)	Dec. 20. Good growth in cultures 1 and 2; medium in good condition. Dec. 21. Good growth; medium in good condition. No. 2 fixed and photographed. Dec. 22. Good growth; medium in good condition.
16	Dec. 22	Washed in Ringer solution for 1 minute, divided into 2 parts, and cultivated in the same medium	Dec. 23. Growing; medium in good condition. Dec. 24. Growing; medium partially liquefied.
17	Dec. 24	Washed in Ringer solution for 1 minute and cultivated in the same medium	Dec. 26. Growing; medium in good condition.
18	Dec. 26	Washed in Ringer solution for 1 minute and cultivated in the same medium	Dec. 27. A few cells; medium in good condition. Dec. 28. Growing; medium in good condition. Dec. 29. Growing; medium in good condition.
19	Dec. 29	Washed in Ringer solution for 1 minute and cultivated in the same medium	Dec. 30. A few cells; medium in good condition. Dec. 31. Growing; medium in good condition.
20	Dec. 31	Washed in Ringer solution for 1 minute and cultivated in the same medium	Jan. 2. Growing; medium in good condition.
21	(1914) Jan. 2	Washed in Ringer solution for 1 minute and cultivated in the same medium	Jan. 3. No growth; medium in good condition. Jan. 4. A few cells.
22	Jan. 4	Washed in Ringer solution for 1 minute and cultivated in the same medium	Jan. 5. A few cells; medium in good condition. Jan. 6. Slow growth; medium slightly liquefied.
23	Jan. 6	Washed in Ringer solution for 1 minute and cultivated in the same medium	Jan. 7. No growth. Jan. 8. A few scattered cells; medium in good condition. Jan. 9. Growing slowly; medium slightly liquefied.

TABLE I.—*Concluded.*

Passage.	Date (1914).	Treatment of culture.	Observations.
24	Jan. 9	Washed in Ringer solution for 1 minute and cultivated in the same medium	Jan. 10. Slight growth; medium in good condition. Jan. 11. No increase in growth; medium in good condition.
25	Jan. 11	Washed in Ringer solution for 1 minute and cultivated in the same medium	Jan. 12. No growth. Jan. 13. No growth. Jan. 14. Discarded.

(second passage). Culture 4 showed a few proliferating cells after seventy-two hours' incubation, and the medium was in good condition. The culture was discarded. After twenty-four hours' incubation in the second passage culture 3 showed growth with no liquefaction. In forty-eight and seventy-two hours growth had increased, but the medium was partially liquefied. The culture was transferred into fresh medium (third passage) and showed a few cells which had spread out into the medium from the central fragment. After seventy-two hours there was no increase in the number of cells, and the medium had liquefied. The culture was transferred into fresh medium (fourth passage), and after forty-eight hours good growth was observed. The medium was in good condition. The fifth passage into fresh medium was made in the same manner as in the previous passage. After twenty-four hours the medium was totally liquefied and no growth was observed. The fragment was transferred to fresh medium, but after 24, 48, and 72 hours no growth developed. The culture was discarded.

RESULTS.

Two experiments were made in which fragments from human sarcomatous tissue were cultivated. It was possible to keep cultures of such tissue in a condition of active life *in vitro* for several generations.

During the first twenty-four hours of incubation there was usually no evidence of cell proliferation, and slight liquefaction around the primitive fragments. When no liquefaction occurred, growth of new cells manifested itself after forty-eight hours. Twenty-four hours after passage into fresh medium (first passage), cell proliferation was observed in those cultures which showed no evidence of

growth when first cultivated. In comparison with human connective tissue, the rate of growth was practically the same in the beginning, but a gradual decrease in the activity and extent of cell proliferation was observed as the length of time increased during which the culture was carried through successive passages. Microscopic examination of the first outgrowth of cells showed the presence of large, round, as well as elongated and ramified cells. In subsequent passages the round cells were no longer to be identified, and the elongated, ramified variety only were observed. The morphological characteristics of these cells did not appear to differ from the cells present in cultures of normal human connective tissue. Preparations stained with Giemsa stain showed the large round cells as having a densely stained cytoplasm with from one to two nuclei and a regular outline. The elongated and ramified varieties showed no difference in comparison with those present in cultures of human connective tissue, with the exception that no mitotic figures were observed. Figure 3 shows a few of the peripheral cells in a culture of sarcomatous tissue which had been carried through twelve passages.

One culture was stained which was growing actively in its twelfth passage (twenty-one days). This culture is shown in figure 1, the area of cell proliferation being that which developed in the twelfth passage during forty-eight hours' incubation. One other culture was carried through for twenty-four passages, that is, fifty-two days. It was possible to divide this culture in its fifteenth passage, making two, and after forty-eight hours' incubation one of these cultures was fixed. Figure 2 shows almost the entire culture.

Sarcomatous tissue grew as well during a few days as normal connective tissue. Afterwards the rate of growth became less rapid and the tissue could not be kept alive for more than fifty-two days, while normal human tissue could be kept for sixty-eight days.

These differences may be due to technical factors, but they may also be the result of the nature of the tissue itself. In his attempts at keeping Rous sarcoma in a condition of permanent life *in vitro*, Carrel[6] observed that after a few generations the rate of growth became less rapid than the rate of growth of connective tissue. In

[6] Carrel, A., *Jour. Exper. Med.*, 1912, xv, 516.

other experiments with rat sarcoma and normal rat connective tissue, cultivated in guinea pig plasma, Carrel also observed the same differences. The writers observed the same phenomena when rat sarcoma and normal heart tissue of the rat were cultivated in chicken plasma.

The results obtained show that it is possible to cultivate *in vitro* fragments of human sarcomatous tissue for several generations, and that the method employed may prove of value in the study of the growth of human malignant tumor.

EXPLANATION OF PLATES.

Plate 15.

Fig. 1. 12th passage of human sarcomatous tissue. The preparation shows the area of cell proliferation that developed during forty-eight hours' incubation. Giemsa stain.

Plate 16.

Fig. 2. 15th passage of human sarcomatous tissue. The photograph shows the growth obtained forty-eight hours after passage into fresh medium. Fixed specimen.

Plate 17.

Fig. 3. High power magnification of some of the peripheral cells present in the same preparation shown in figure 1.

THE REMOVAL OF CALCIUM FROM THE BLOOD BY DIALYSIS IN THE STUDY OF TETANY.*

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On the appearance of the paper by Abel, Rowntree, and Turner[1] describing a method by which diffusible substances might be removed from the circulating blood by dialysis, it occurred to us that we might attack the question of the relation of calcium salts to tetany in a new way.

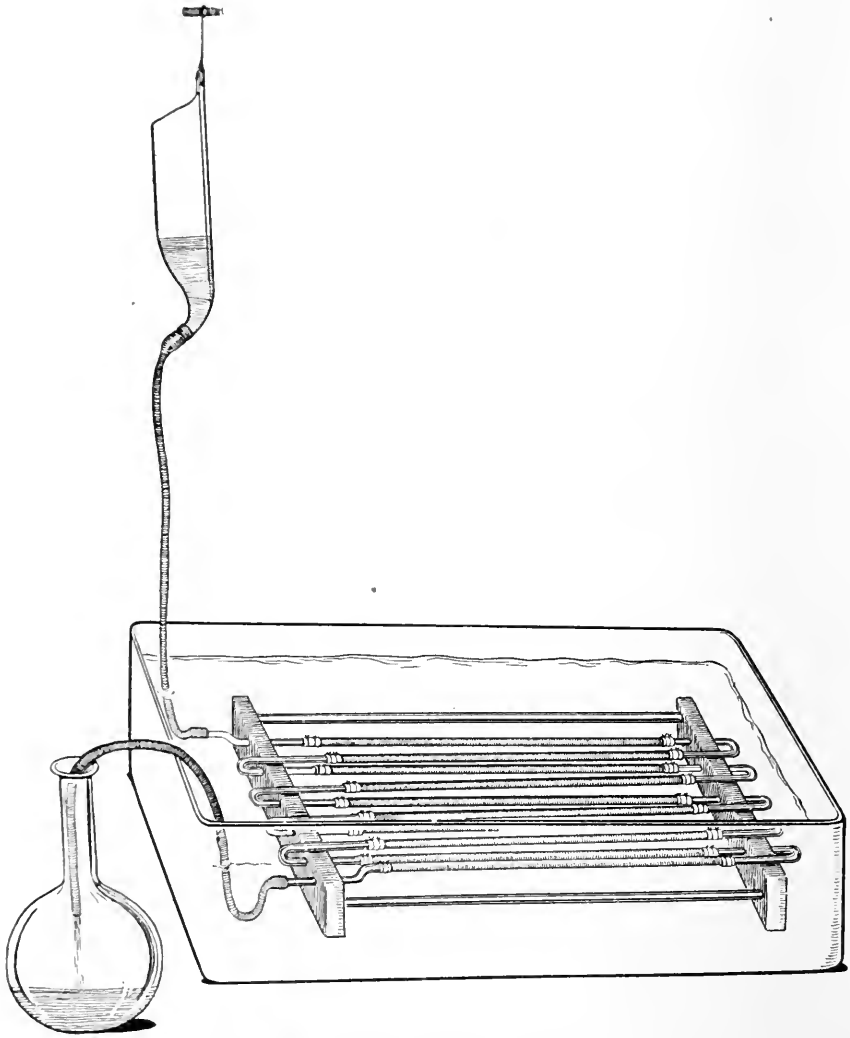
At first we thought that we might be able to produce tetany by mechanically removing calcium from the blood by allowing it to circulate through celloidin tubes immersed in an isotonic solution containing no calcium. We therefore constructed a machine somewhat like Abel's, and made a number of experiments with this object in view, using a fluid put together according to Abderhalden's analysis of the inorganic constituents of the dog's blood, but without calcium. We varied this fluid by substituting phosphates for chlorides, with the idea that by removing chlorides also we might still further reduce the proportion of soluble calcium. We used defibrinated blood instead of salt solution in starting the current, and finally defibrinated all the animal's blood. The results were practically negative, and we abandoned the method because it seemed that we could attain our end better in another way without exposing the animal to an operation which must be prolonged over many hours.

We adopted a method which consisted in passing a quantity of defibrinated blood from another dog through a modified dialyzing apparatus (text-figure 1) for many hours until, as we could ascer-

* Received for publication, June 2, 1914.

[1] Abel, J. J., Rowntree, L. G., and Turner, B. B., *Jour. Pharmacol. and Exper. Therap.*, 1914, v, 275.

tain by chemical analysis, the amount of calcium was greatly reduced and had passed over into the dialyzing fluid. It was then



TEXT-FIG. 1. Dialysis apparatus of celloidin tubes in series.

proposed to run the blood into the veins of a normal animal after an equal amount had been removed from the carotid artery, but even before this was done, it was thought best to ascertain the effect of

the dialyzed blood upon the nerves of an isolated extremity by perfusion, according to the method previously employed[2] in studying the blood of animals in tetany. A number of experiments made in this way gave the following results.

Dog 1,420.—Normal extremity. Perfusion started at 3.50 P. M. with blood dialyzed against calcium-free fluid.

	2.00	3.30	3.50	4.00	4.10	4.20	4.40	4.50
KC[3]	0.2	0.05	Perfusion started	0.05	0.05	0.05	0.1	0.2
KO	—[4]	—		0.6	1.8	1.2	1.6	2.4
AC	0.5	1.0		0.2	0.5	0.6	0.4	1.2
AO	1.4	0.4		0.5	0.5	0.6	0.4	1.2

Blood used in perfusion contained calcium per 1,000 c.c. as follows:

Before dialysis.	After dialysis.	After perfusion.
0.085	0.0465	0.080

Dog 1,421.—Normal isolated extremity. Perfusion started at 4.15 P. M. in the same way.

	3.50	4.00	4.15	4.20	4.30	4.45	5.00	5.15	5.30
KC	0.4	0.6	Perfusion	0.2	0.4	0.4	0.4	0.4	0.4
KO	—	—		1.6	2.8	1.8	1.8	2.6	1.6
AC	1.0	1.6		1.2	1.0	1.0	1.2	1.0	1.0
AO	1.2	2.0		1.2	1.0	1.0	1.0	0.8	1.0

Dog 1,427.—Normal leg perfused first with normal then with dialyzed blood.

	8.45	9.00	9.30	9.45	9.50	10.00	10.10	10.15	10.20	10.30	10.40	10.50	11.00	11.10	11.20	11.30	11.35	11.50
KC	0.3	0.4	Perfusion complete Normal blood	0.6	0.8	0.8	1.0	1.0	0.6	0.6	0.6	0.6	0.8	0.8	1.0	1.0	2.0	1.2
KO	—	—		—	—	—	—	—	4.4	3.6	4.0	3.4	3.4	—	—	—	—	—
AC	1.6	1.6		2.4	2.4	2.4	2.8	2.4	2.0	2.0	2.0	2.0	2.2	2.2	2.4	3.4	3.4	4.0
AO	1.8	2.4		—	4.6	4.6	3.2	2.6	2.0	2.0	2.0	2.0	2.0	1.4	2.4	2.2	2.6	4.0

Dialyzed blood Normal blood

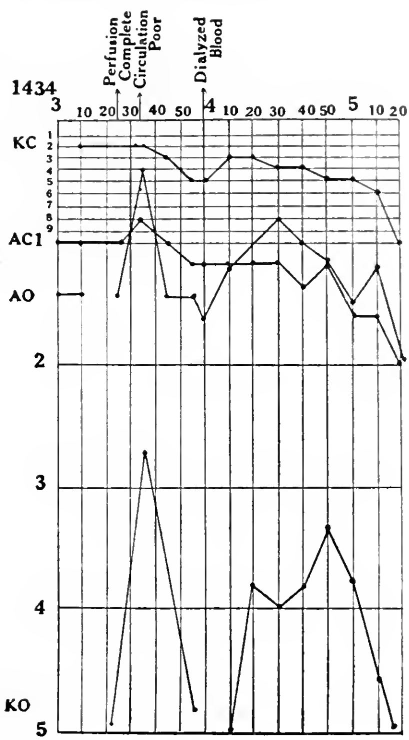
[2] MacCallum, W. G., *Mitt. a. d. Grenzgeb. d. Med. u. Chir.*, 1913, xxv, 941.
[3] KC = kathode closing; AO = anode opening, etc.
[4] In all the tables — indicates negative up to 5 milliamperes.

It will be seen from these tables that the results of perfusion with dialyzed blood were not especially striking, nor do they convince one that the alterations in excitability of the nerves are strictly due to the withdrawal of calcium. From long previous experience, although it is known that a rise in excitability of a brief and temporary nature can come about from inadequacy of the circulation and that the great fall at the end of each curve is doubtless due to this or to some direct injury of other kinds, still it is also known that with a good circulation with normal blood the excitability will remain almost constant. These curves were especially inconclusive in that while a visible contraction could be elicited with weak currents, strong shocks gave only about the same muscular jerk. Instead of a brisk sharp contraction the foot moved lazily, often twisting in a peculiar way, and after the perfusion with dialyzed blood had gone on for only a short time all peripheral contractions disappeared and the shock produced only jerking of the thigh muscles behind the electrode. These are inevitable signs of approaching death of the nerve, impressing us rather as the consequences of some sort of poisoning, and in every case the dialyzed blood was quickly exchanged for normal blood, sometimes with a return of the excitability.

Discouraged by these results, we determined to add the normal amount of calcium to our dialyzing fluid, which at this time had the following composition:

Sodium chloride	60.0
Disodium phosphate	28.5
Potassium chloride	4.0
Magnesium chloride	2.87
Sodium bicarbonate	5.0
Dextrose	10.9
Water	10,000

It became apparent at once, as we might have foreseen, that the calcium is precipitated by the phosphates present. Phosphates were therefore left out and calcium was added. After dialysis with this fluid, perfusion of the leg gave almost the curve which one would expect with normal blood.



TEXT-FIG. 2. Perfusion of leg with blood dialyzed against fluid containing all inorganic constituents of blood including calcium.

Dog 1,434.—(Text-figure 2.) Normal leg perfused with blood dialyzed against a fluid containing calcium but no phosphates.

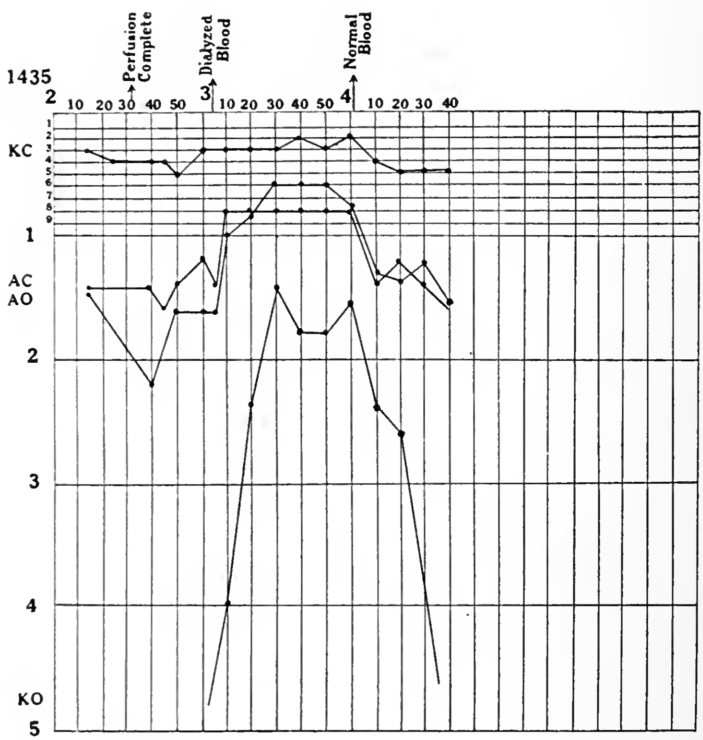
Sodium chloride	60.0
Sodium bicarbonate	5.0
Potassium chloride	4.0
Magnesium chloride	2.3
Dextrose	11.0
Calcium lactate	6.0
Water	10,000

	3.10	3.25	3.35	3.45	3.55	4.00	4.10	4.20	4.30	4.40	4.50	5.00	5.10	5.20
KC.	0.2	Perfusion	0.2	0.3	0.5	0.5	0.3	0.3	0.4	0.4	0.5	0.5	0.6	1.0
KO.	—		2.8	—	—	—	5.0	3.8	4.0	3.8	3.4	3.8	4.6	6.0
AC.	1.0		0.8	1.0	1.2	1.2	1.2	1.2	1.2	1.4	1.2	1.6	1.6	2.0
AO.	1.4		0.4	1.4	1.4	1.6	1.2	1.0	0.8	1.0	1.2	1.6	1.2	2.0

It will be seen from text-figure 2 that during the perfusion of normal blood a partial clamping off of the supply tube produced a

brief rise in excitability which then passed away when the current was turned on in its full strength.

Therefore, from that point the experiments were begun again with a dialyzing fluid containing neither calcium nor any considerable quantity of the disturbing phosphates. Probably with this fluid the blood lost some of its phosphates, but the difficulty of devising a dialyzing fluid which should remove nothing but calcium from the blood seemed insurmountable.[5]



TEXT-FIG. 3. Perfusion of leg with blood dialyzed against fluid devoid of calcium.

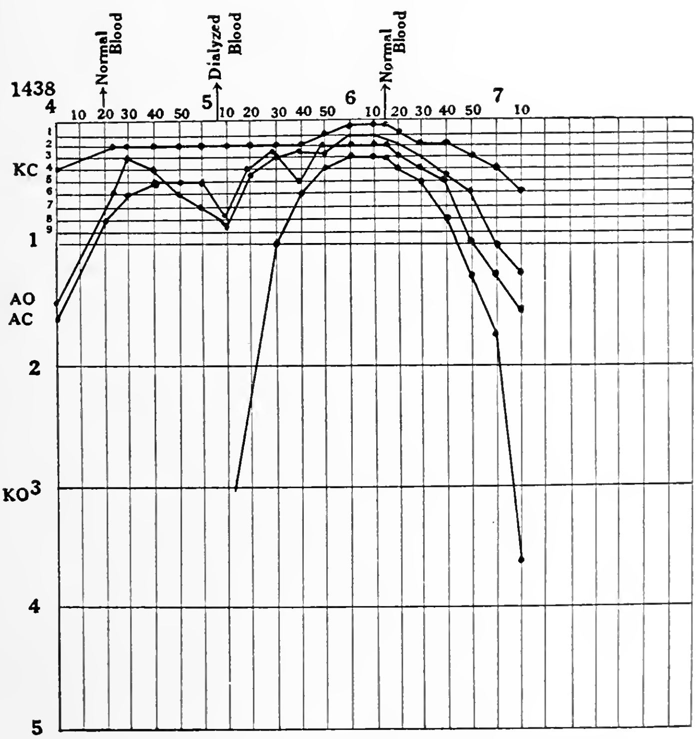
Dog 1,435.—(Text-figure 3.) Normal leg perfused at 2.32 P. M. with normal blood, at 3.01 with dialyzed blood, and at 4.01 again with normal blood. 900 c.c. of normal blood were dialyzed for 7½ hours against 18,000 c.c. of fluid (fluid 3) of the following composition, which was used in all later experiments.

[5] Since writing this paper it has been suggested to us by Dr. A. E. Taylor that as the phosphates in the blood are in non-dialyzable combinations there is little source of error in using a dialyzing fluid without phosphates.

Sodium chloride	60.0
Sodium bicarbonate	5.0
Magnesium chloride	2.30
Potassium chloride	4.0
Dextrose	10.9
Water	10,000

	2.15	2.18	2.32	2.40	2.45	2.50	3.00	3.05	3.10	3.20	3.30	3.40	3.50	4.00	4.10	4.20	4.30	4.40
KC	0.3	0.4	Perfusion complete	0.4	0.4	0.5	0.4	0.3	0.3	0.3	0.3	0.2	0.3	0.2	0.4	0.5	0.5	0.5
KO	—	—		—	—	—	—	—	4.0	2.4	1.4	1.8	1.8	1.6	2.4	2.6	—	4.0
AC	1.1	0.8		1.4	1.6	1.4	1.2	1.4	0.8	0.8	0.8	0.8	0.8	0.8	1.4	1.2	1.4	1.6
AO	1.4	1.0		2.2	2.0	1.6	1.6	1.6	1.0	0.8	0.6	0.6	0.6	0.8	1.4	1.4	1.2	1.6

Dialyzed blood   Normal blood



Text-Fig. 4. Perfusion of leg with blood dialyzed against fluid devoid of calcium.

Dog 1,438.—(Text-figure 4.) Normal leg perfused at 4.20 P. M. with normal blood. This was changed to dialyzed blood at 5.02, and again to normal blood at 6.12. The dialyzed blood was 1,000 c.c. which had been poured through the long celloidin tubules for 7 hours against 24,000 c.c. of the fluid described above.

	4.00	4.20	4.25	4.30	4.40	4.50	5.00	5.05	5.10	5.20	5.30	5.40	5.50	6.00	6.10	6.20	6.30	6.40	6.50	7.00	7.10
KC..	0.4	Perfusion complete	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.1	0.05	0.05	0.1	0.2	0.2	0.3	0.4	0.6
KO..	—		..	—	—	—	—	—	—	—	1.0	0.6	0.4	0.5	0.3	0.4	0.5	0.8	1.2	1.8	3.6
AC..	1.4		0.4	0.6	0.5	0.5	0.5	0.8	0.8	0.4	0.2	0.5	0.2	0.2	0.2	0.3	0.4	0.5	1.0	1.2	1.4
AO..	1.4		0.6	0.3	0.4	0.6	0.7	0.8	0.8	0.4	0.2	0.2	0.2	0.1	0.1	0.2	0.3	0.3	0.6	1.0	1.2

Normal blood　　Dialyzed blood　　Normal blood

On the entrance of the dialyzed blood the leg jerked and moved itself about. Twitchings were evident until about 5.20.

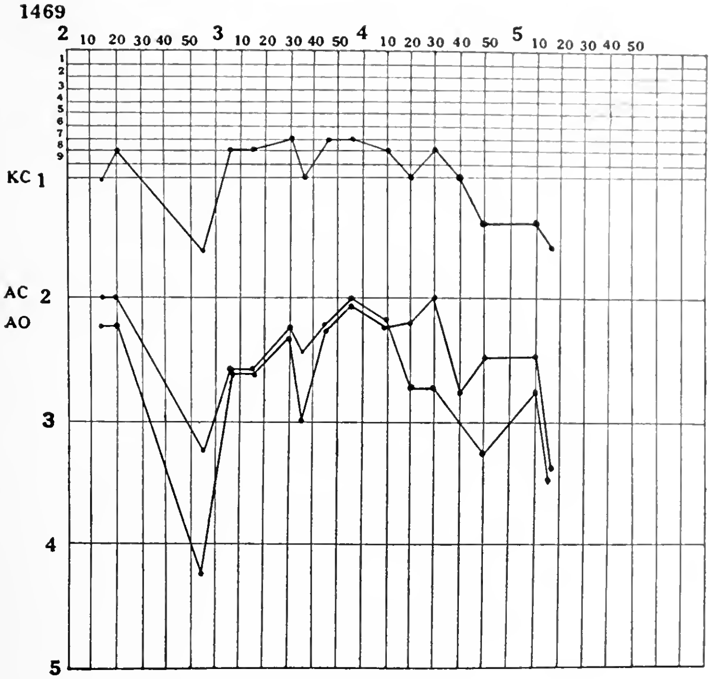
The chemical analysis of the dialyzing fluid before and after dialysis, and of the blood at various points in the experiments, is as follows. The figures show the amount of calcium per 1,000 c.c.

	Dog 1,435.	Dog 1,438.
Dialyzing fluid before dialysis of blood	0.0040	0.0043
Dialyzing fluid after dialysis of blood (1st)	0.0076	0.0076
Dialyzing fluid after dialysis of blood (2d)	0.0048	0.0054
Blood before dialysis	0.0364	0.0442
Blood after dialysis	0.0284	0.0233
Blood after perfusion of leg	0.0212(?)	0.0300

Text-figures 3 and 4 show clearly that perfusion with blood partly deprived of its calcium by dialysis produces great hyperexcitability of the nerves, and further that this must be due solely to the loss of calcium, since exactly the same procedure, changed only by the addition of precisely enough calcium to the dialyzing fluid to prevent any loss or gain of calcium by the blood, brings about no such hyperexcitability of the nerves. This proof may be somewhat strengthened by the addition of another curve made in the same way, except that the dialyzing fluid was so devised as to remove a large part of the chlorides from the blood, leaving the calcium unchanged.

Dog 1,469.—(Text-figure 5.) Normal leg perfused at 2.45 P. M. with normal blood, and at 3.30 with dialyzed blood. Dialysis was of 800 c.c. of normal blood for 6½ hours against 30,000 c.c. of fluid 4 of the following composition:

Sodium acetate	50.0
Sodium nitrate	40.0
Sodium bicarbonate	10.0
Magnesium chloride	2.3
Potassium nitrate	5.25
Calcium lactate	5.0
Dextrose	20.0
Water	10,000



Text-Fig. 5. Leg perfused with blood dialyzed against a chloride-free fluid.

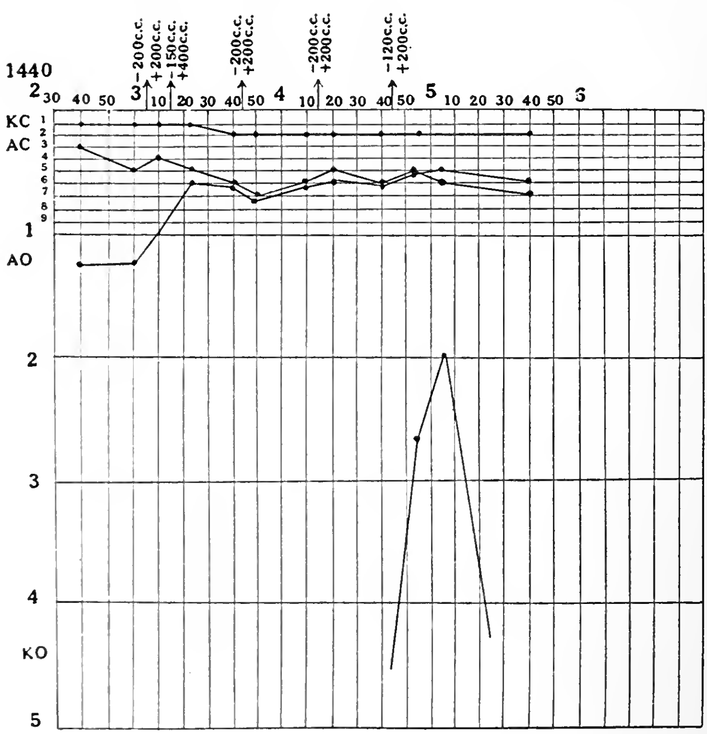
	2.15	2.20	2.45		2.55	3.05	3.15	3.30	3.35	3.43	3.55	4.10	4.20	4.30	4.40	4.50	5.10	5.15
KC...	1.0	0.8	Perfusion	Normal blood	1.6	0.8	0.8	0.7	1.0	0.7	0.7	0.8	1.0	0.8	1.0	1.4	1.4	1.6
KO...	—	—			—	—	—	—	—	—	—	—	—	—	—	—	—	—
AC...	2.0	2.0			3.2	2.6	2.6	2.2	2.4	2.2	2.0	2.2	2.8	2.8	3.0	3.2	2.8	3.4
AO...	2.2	2.2			4.2	2.6	2.6	2.2	3.0	2.2	2.0	2.2	2.2	2.0	2.8	2.6	2.6	3.4

Dialyzed blood

It is seen from text-figure 5, in which the variations are so slight, that hyperexcitability of the nerves is not produced by long perfusion, even with blood greatly altered by dialysis, so long as the dialysis has not removed the calcium. When the calcium is diminished, however, the effect in producing hyperexcitability is prompt and persists as in the case of tetany blood so long as blood altered in this way is passing, giving way to normal conditions as soon as normal blood is again circulated.

When an isolated extremity is perfused the influence of the undiluted dialyzed blood must be felt by the nerves, since there is but little tissue from which calcium or other constituents could be

absorbed in the passage of the blood. We thought, therefore, that this might be the most trustworthy expression of the nature of the influence of the dialyzed blood. Nevertheless we attempted to introduce dialyzed blood in quantity into the veins of an animal, realizing that it would at once meet with an abundant source of calcium in the tissues, but hoping to circulate it so rapidly as to surprise the nervous system and produce some hyperexcitability not only of the peripheral nerves but of the ganglion cells. We planned not only to wash out all the animal's blood with dialyzed blood but to replace that, too, with fresh dialyzed blood. Indeed after one or two experiments we even circulated fresh dialyzed blood through the whole animal, so that if the tissues were to supply calcium to all the new blood presented to them they must furnish enough to bring back to normal the calcium content of five or six times as much blood as was possessed by the animal before. At the beginning of each



TEXT-FIG. 6. Repeated bleeding from carotid with replacement of blood dialyzed until poor in calcium.

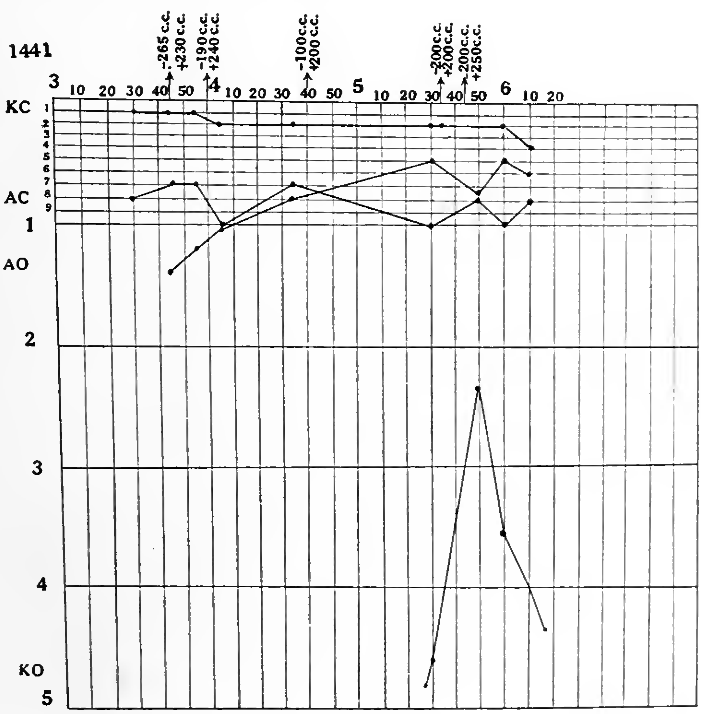
experiment the parathyroids of the animal were removed. The protocols are as follows:

Dog 1,440.—(Text-figure 6.) Normal animal bled at intervals, dialyzed blood being returned. Thyroids and parathyroids removed at 4.35 P. M. No tetany developed.

	2.40	3.00	3.10	3.25	3.40	3.50	4.10	4.20	4.45	4.55	5.05	5.40
KC.	0.1	0.1	0.1	0.1	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
KO.	—	—	—	—	—	—	—	—	—	2.6	2.0	—
AC.	0.3	0.5	0.4	0.5	0.6	0.7	0.6	0.5	0.6	0.5	0.6	0.7
AO.	1.2	1.2	1.0	0.6	0.6	0.7	0.6	0.6	0.6	0.5	0.5	0.6

Bled	200 c.c.	150 c c.	200 c.c.	120 c c.
Returned dialyzed blood	200 c.c.	400 c c.	200 c.c.	200 c.c.

In this experiment 1,350 c.c. of blood dialyzed for 7 hours against 30,000 c.c. of fluid 3 were used in replacing normal blood. Effect very slight.



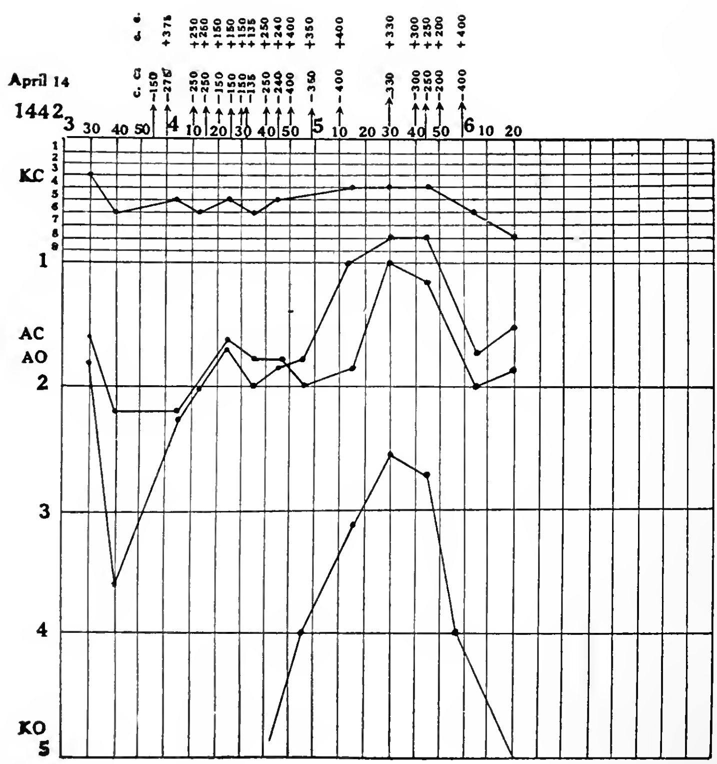
TEXT-FIG. 7. Normal animal. Blood replaced at intervals with dialyzed blood.

Dog 1,441.—(Text-figure 7.) Normal animal, blood at intervals replaced with dialyzed blood. 1,300 c.c. of blood dialyzed for 7½ hours against 26,000 c.c. of fluid 3. Parathyroids were removed just before the experiment started.

	3.30	3.45	3.55	4.05	4.35	5.30	5.50	6.00	6.10
KC .	0.1	0.1	0.1	0.2	0.2	0.2	0.2	0.2	0.4
KO .	—	—	—	—	—	4.6	2.4	3.6	4.0
AC .	0.8	0.7	0.7	1.0	0.7	1.0	0.8	1.0	0.8
AO .	1.4	1.4	1.2	1.0	0.8	0.5	0.8	0.5	0.6

Bled	265 c.c.	190 c.c.	200 c.c.	200 c.c.
Returned dialyzed blood	230 c.c.	240 c.c.	200 c.c.	250 c.c.

There is general twitching; no definite tetany; much vomiting.



TEXT-FIG. 8. Normal animal through which dialyzed blood was actually circulated.

Dog 1,442.—(Text-figure 8.) Normal animal through whose blood vessels dialyzed blood was actually circulated, as shown in the chart. Parathyroids first removed.

	3.20	3.40	4.05	4.12	4.25	4.35	4.45	4.55	5.15	5.30	5.45	6.05	6.20
KC...	0.3	0.6	0.5	0.6	0.5	0.6	0.5	0.5	0.4	0.4	0.4	0.6	0.8
KO...	—	—	—	—	—	—	—	4.0	3.2	2.6	2.8	4.0	5.0
AC...	1.6	2.2	2.2	2.0	1.6	1.8	1.8	2.0	1.8	1.0	1.2	2.0	1.8
AO...	1.8	3.6	2.2	2.0	1.6	2.0	1.8	1.8	1.0	0.8	0.8	1.8	1.6

Bled	150 c.c.	275 c.c.	250 c.c.	250 c.c.	150 c.c.	150 c.c.	135 c.c.	250 c.c.	240 c.c.	400 c.c.	350 c.c.	400 c.c.	330 c.c.	300 c.c.	250 c.c.	200 c.c.	400 c.c.
Returned dialyzed blood	375 c.c.		250 c.c.	250 c.c.	150 c.c.	150 c.c.	135 c.c.	250 c.c.	240 c.c.	400 c.c.	350 c.c.	400 c.c.	330 c.c.	200 c.c.	250 c.c.	200 c.c.	400 c.c.

1,800 c.c. of blood dialyzed for 7 hours against 30,000 c.c. of fluid 3 were used. Each part of this probably circulated two and one half times. Vomiting; no definite twitching; collapse and death.

From these experiments it seems clear that it is impossible in a short time to reduce the calcium content of the blood circulating through the whole body or to keep it reduced to such a degree as to bring about a tetany-like hyperexcitability of the nerves. There is too abundant a source for fresh calcium in the various tissues, including the bones, and the blood quickly replenishes itself. It might seem possible to repeat this process during several days and thus imitate the latent period which follows extirpation of the parathyroids, but in this case it would be necessary to extirpate those glands, when tetany would arise of itself, since otherwise the blood would rapidly replenish its calcium, under their influence, from the skeleton. Indeed, the rapidity with which calcium enters the circulating dialyzed blood from the tissues is shown by comparison of the analyses of samples taken before and after its circulation in the body.

In dog 1,441 the blood contained per 1,000 c.c.:

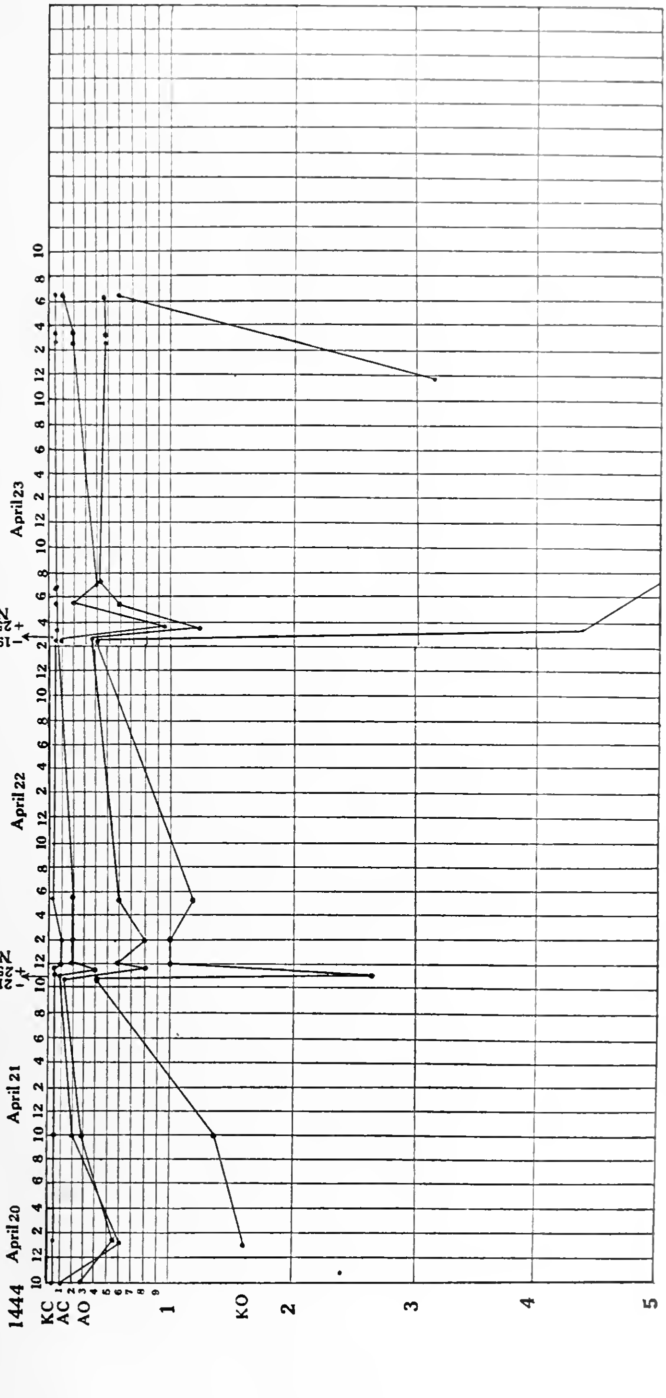
Before dialysis	0.0485
After dialysis	0.0190
After circulation for about 2 hours	0.0381

It remains a question whether, if one could continue this depletion over several days, it would be possible to produce such changes in excitability as are seen in tetany, and in fact this was our original problem. It leaves one with the impression that the parathyroid secretion must govern the conversion of a non-dialyzable and useless combination of calcium into a dialyzable form which is essential for the control of the excitability of the nerves, but which is lost

in the excreta and not newly formed in the absence of parathyroid secretion.

Although we had learned that it is possible by perfusion with calcium-poor blood to produce hyperexcitability of the nerve, it remained to bring this fact into direct relation with the condition in tetany, for it might be objected that there the mechanism is a different one. This we thought would be attained if we could show that this dialyzed blood had lost the power possessed by normal blood of curing tetany on transfusion. It is well known that if an animal in tetany, after the removal of the parathyroids, be bled and the blood replaced with normal blood, the excitability of the nerves falls, and twitchings disappear for a time. Dialyzed blood poor in calcium has little or nothing of this effect, although if the dialyzed blood be not so poor in calcium as that of the animal in tetany, it will lower the excitability somewhat. If in an animal in tetany we replace the blood with dialyzed blood and find no change in the symptoms nor lowering of the excitability, we may remove that blood and replace it with normal blood which does stop the twitching and lower the excitability.

But if, although the excitability is not lowered by the dose of dialyzed blood, we do nothing further, the excitability is found to be lowered the next day or after some hours. To explain this we have imagined that while in dialysis we remove the calcium from the normal blood, we may not remove the parathyroid secretion which on being injected into a dog in tetany after a time causes the appearance of dialyzable calcium in the blood and goes far to relieve temporarily the tetany. It is on this basis that it seems hopeless to attempt to produce a condition resembling tetany by the repeated replacement of the blood over a period of days with normal blood dialyzed until it is poor in calcium, because at the same time we furnish parathyroid secretion which ensures the continual withdrawal of calcium from the tissues. It is true that we might dialyze tetany blood, but even then having a blood free from parathyroid secretion and poor in calcium we have no animal in which to make evident the effect of pure lack of calcium, for the animal with intact parathyroids will continually frustrate this object, and the animal deprived of parathyroids will develop tetany during the experiment.



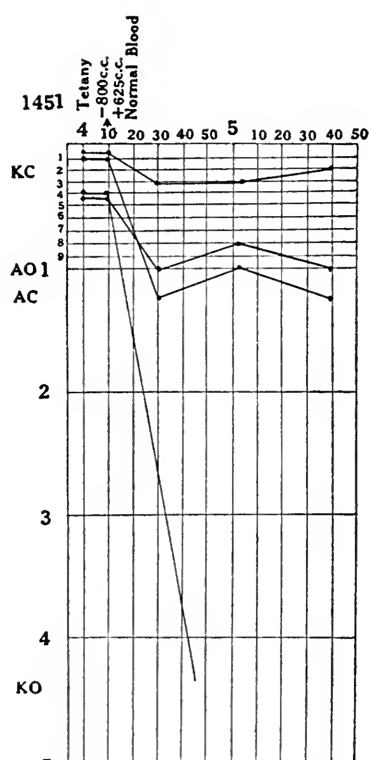
TEXT-FIG. 9. Tetany. Bled on two occasions; normal blood replaced. Transient effect.

ILLUSTRATIVE PROTOCOLS.

Dog 1,444.—(Text-figure 9.) Tetany developing 2 days after parathyroidectomy. Bled on two occasions, normal blood being replaced. Transient relaxation and lowering of excitability.

	April 20.		April 21.						April 22.				April 23.		
	10.00	1.45	10.00	11.15	11.40	12.00	2.00	5.45	2.30	2.55	5.30	6.35	2.20	3.10	6.30
KC	0.05	0.05	0.05	0.05	0.05	0.1	0.1	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
KO	.....	1.6	1.4	0.4	2.6	1.0	1.0	1.2	0.4	4.4	—	—	—	—	0.6
AC	0.1	0.6	0.2	0.05	0.4	0.2	0.2	0.2	0.05	1.0	0.2	0.4	0.2	0.2	0.1
AO	0.3	0.6	0.3	0.1	0.8	0.6	0.8	0.6	0.4	1.2	0.6	0.4	0.5	0.5	0.5

Bled 215 c.c. 190 c.c.
Returned normal blood 250 c.c. 250 c.c.



TEXT-FIG. 10. Tetany. Effect of bleeding and replacing normal blood.

Dog 1,451.—(Text-figure 10.) Violent tetany 24 hours after parathyroidectomy. Bled 800 c.c., and this loss was replaced by 625 c.c. of normal blood. Immediate relaxation and lowering of excitability of nerves.[6]

[6] A curious phenomenon was observed in these cases in which nearly all the

	April 26.					April 27.	April 28.
	4.00	5.00	5.20	5.55	6.30	11.00	12.15
KC.......	0.05	Blood re-placed by normal	0.3	0.3	0.2	0.1	0.05
KO.......	0.4		—	—	—	—	0.3
AC.......	0.1		1.2	1.0	1.2	1.2	0.05
AO.......	0.4		1.0	0.8	1.0	1.2	0.2

Bled 800 c.c. 425 c.c.
Returned normal blood 625 c.c. Returned dialyzed blood 400 c.c.

This animal developed tetany again on April 28 and was bled 425 c.c., 400 c.c. of dialyzed blood being replaced. There was no recovery from the spasm brought on by the bleeding, and death followed rapidly. This case forms an example of the contrast between the effects of introducing normal and dialyzed blood.

Dog 1,445.—Moderate tetany 2 days after parathyroidectomy. At 2.23 P. M. bled 150 c.c. Blood replaced by 150 c.c. of blood dialyzed for 5 hours against 10,000 c.c. of fluid 3. At 3.20 bled 150 c.c., and replaced 150 c.c. of normal blood. At 10.30 bled 200 c.c., and replaced 250 c.c. of normal blood.

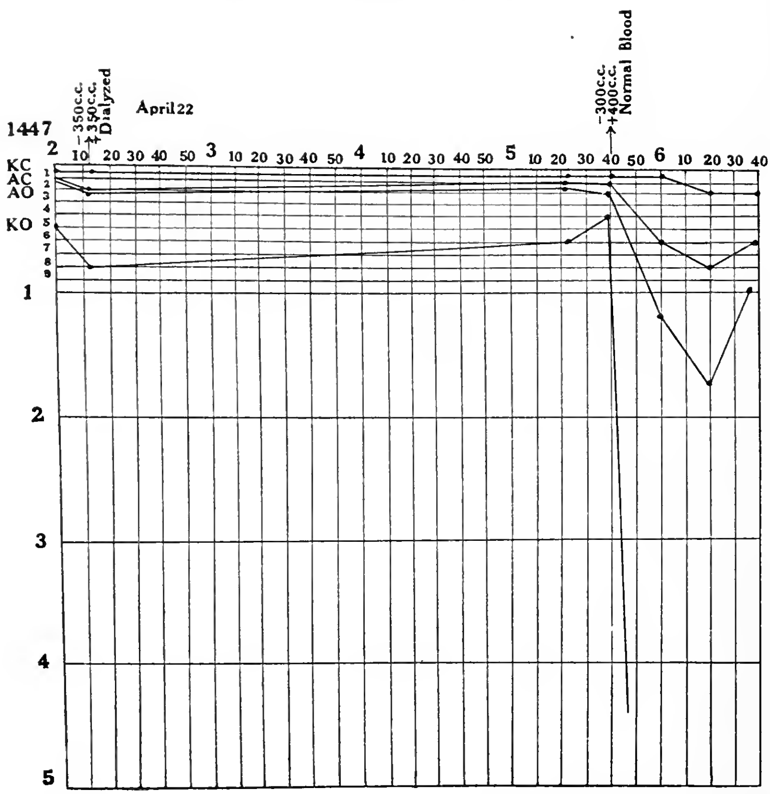
	April 20.									April 21.						
	1.30	1.45	2.20	2.30	2.40	3.00	3.20	3.45	4.25	10.00	10.45	11.00	11.15	12.00	2.00	5.45
KC.	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.1	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
KO.	0.2	0.3	0.3	0.3	0.4	0.3	0.6	1.4	0.6	0.2	0.4	0.4	0.4	—[7]	2.0	1.4
AC.	0.05	0.1	0.05	0.05	0.05	0.05	0.1	0.2	0.1	0.05	0.05	0.1	0.1	0.1	0.2	0.3
AO.	0.2	0.3	0.3	0.3	0.4	0.3	0.4	0.5	0.3	0.3	0.4	0.5	0.4	0.4	0.3	0.3

Bled 150 c.c. 150 c.c. 200 c.c.
Returned dialyzed blood 150 c.c. Returned normal blood 150 c.c. 200 c.c.

Dog 1,447.—(Text-figure 11). Violent tetany at 2 P. M. Bled 350 c.c. which were replaced with 350 c.c. of blood from a lot of 600 c.c. which had been dialyzed for 4½ hours against 12,000 c.c. of fluid 3. Change in character of electric reaction, but no marked change in excitability. Contraction slow, no rigidity. Tongue tremor and twitchings continue. At 5.45 bled 300 c.c., and replaced 400 c.c. of normal blood. Complete relaxation and return of excitability to normal.

blood was removed, at the moment before the normal blood was allowed to run in. Although twitching may have been slight when the bleeding began, the most violent contractions and extreme rigidity appeared during the extreme anemia, to disappear at once when the other blood entered the veins. Probably this is due to temporary asphyxia of the nerves.

[7] Negative up to 3 milliamperes.



TEXT-FIG. 11. Tetany. Bleeding on two occasions. Dialyzed blood replaced without effect. Normal blood reduces excitability.

	April 22.										
	11.45	2.00	2.15	2.55	5.25	5.40	6.00	6.20	6.45	9.00	12.00
KC..	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.2	0.2	Quiet; no tongue tremor	Twitching; tremor in tongue.
KO..	0.5	0.5	0.8	Tongue and muscle tremor	0.6	0.4	—	—	—		
AC..	0.1	0.1	0.2		0.1	0.1	0.6	0.8	0.6		
AO..	0.1	0.1	0.2		0.1	0.2	1.2	1.8	1.0		

Bled 350 c.c. 300 c.c.
Returned dialyzed blood 350 c.c. Returned normal blood 400 c.c.

Dog 1,452.—No tetany 4 days after parathyroidectomy. Moderately high excitability. Bled 315 c.c., replaced by 330 c.c. of blood dialyzed 7 hours against fluid 3, $\frac{430 \text{ c.c. blood}}{10{,}000 \text{ c.c. fluid}}$. Animal much depressed and vomited. Excitability a

little lowered, and this lowering continued and increased for the next two days. Then bled 300 c.c., which were replaced by 300 c.c. of blood dialyzed for 6 hours, $\frac{400 \text{ c.c.}}{15{,}000 \text{ c.c.}}$. No marked change in excitability.

	April 29.					May 1.				
	5.00	5.25	5.35	5.50	6.05	2.00	5.00	5.40	5.50	6.25
KC..........	0.05	0.2	0.2	0.2	0.1	0.2	0.1	0.2	0.2	0.1
KO..........	1.8	2.6	2.0	2.0	2.0	—	—	—	—	—
AC..........	0.4	0.8	0.8	1.2	1.0	1.4	1.2	1.2	1.2	1.2
AO..........	0.5	0.8	0.8	1.0	0.8	2.0	1.4	1.4	1.6	2.0
Bled		315 c.c.					300 c.c.			
Returned dialyzed blood		330 c.c.					300 c.c.			

We made a number of analyses of the blood of animals in tetany, of the dialyzed normal blood used to replace their own, of the normal blood, and of the normal or dialyzed blood removed again after it had circulated for a time in the animal, thinking that a comparison of these figures might throw some light on what occurred in the body to influence the tetany. But such figures would be difficult to interpret because they would change continually with the time the new blood was allowed to stay in the body, first through the process of obtaining a balance with the tissues, and then through the same process that disturbed the composition of the blood to produce tetany. It might be expected then that thoroughly dialyzed blood left in a tetany animal would become richer in calcium for awhile, although normal blood introduced in the same way would steadily lose calcium until it became very poor indeed, when another attack of tetany might come on, as in dog 1,451.

If tetany blood be dialyzed under exactly the same conditions as normal blood, it still loses a proportionate amount of its calcium, which would perhaps show that it is not especially the loss of a diffusible calcium as contrasted with a non-diffusible form which is important in producing tetany. The blood of dog 1,451 taken during tetany contained 0.0287 of a gram of calcium per 1,000 cubic centimeters. 500 cubic centimeters dialyzed for 5 hours against 12,000 cubic centimeters of fluid 3 then contained 0.0114 of a gram per 1,000 cubic centimeters.

CONCLUSIONS.

We may devise a fluid containing practically all the inorganic diffusible constituents of the blood except calcium, and use it to dialyze normal blood in such a way as to remove from it a large part of its calcium. The dialyzed blood when perfused through an isolated extremity produces an extreme hyperexcitability of the nerves quite like that observed in tetany. Since perfusion with blood dialyzed in precisely the same way against a fluid of the same composition, but containing calcium in the proportion found in the normal blood, causes no hyperexcitability of the nerves, it is evident that the hyperexcitability is due to the lack of calcium. This effect can be attained in only a slight degree by replacing the blood of a whole animal with the dialyzed blood, since under the conditions of the experiment the tissues cannot be sufficiently depleted of their calcium. It seems probable that the parathyroid secretion is not removed by dialysis, but is returned to the body with the dialyzed blood.

To bring this result into relation with the condition in tetany following parathyroidectomy, animals in tetany were bled and the blood was replaced in one case with normal blood, in the other with dialyzed blood poor in calcium. The normal blood immediately relieves the tetany and lowers the excitability, while the dialyzed blood does not. We therefore believe that this is a further proof that in the tetany of parathyroidectomy also the twitching and hyperexcitability of the nerves is due to lack of calcium in the blood and tissues.

THE EXPERIMENTAL PRODUCTION OF NECROSIS OF THE LIVER IN THE GUINEA PIG.*

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The starting point for our experiments was the observation that intravenous injections of various substances, as leech extract, colloidal solutions of metals, casein, and nucleoproteid caused an inhibition in the growth of mouse tumors.[1] In order to determine the mechanism through which these substances act, we studied their actions on the organs of guinea pigs, and we noticed that a single intravenous injection of solutions of various proteids and of hirudin apparently caused necrosis in the liver of the injected animals. In the mouse, on the other hand, we did not observe this effect on the liver. Observations concerning the experimental conditions under which necrosis of the liver is produced in the guinea pig are complicated through the apparently spontaneous occurrence of necrosis in the liver of these animals.

Mallory,[2] studying necrosis in the human liver, reports on some experiments in the guinea pig in which he found that various experimental procedures, as massage of the spleen, pressure exerted on the liver, subcutaneous injection of diphtheria toxin, or the passing of a galvanic current through the abdomen of an animal, apparently caused necrosis in the liver. He appears doubtful, however, as to the interpretation of these observations. He noticed this necrosis two hours as well as twenty-four hours after the experimental interference, and furthermore found necrosis in untreated control animals. He comes, therefore, to the conclusion that the guinea pig is entirely unsuited for the study of necrosis of the liver. Inasmuch as no data were given in Mallory's work which make possible a comparison between the number and extent of necroses of the

* Received for publication, May 18, 1914.

[1] Loeb, L., and Fleisher, M. S., *Jour. Am. Med. Assn.*, 1913, lx, 1857.

[2] Mallory, F. B., *Jour. Med. Research*, 1901, vi, 264.

TABLE I.

	Injected with 10 c.c. of 1⅔% solution of casein.	Injected with 2½ c.c. of 15% solution of serum globulin.	Injected with 2 c.c. of 15% solution of nucleoproteid.	Injected with 4–8 c.c. of ¼% solution of hirudin.	Injected with 10 c.c. of colloidal copper.	Injected with 10 c.c. of distilled water.	Injected with 5 c.c. of 15% solution of ovalbumin.	Injected with 3 c.c. of 5% solution of protamin.	Injected with 10 c.c. of 5% solution of starch.	Injected with strophanthin 0.039 mgm. in 10 c.c. of water.	Controls uninjected.
No. of animals....	87	27	51	43	53	24	16	20	21	20	297
No lesion.........	15 (17%)	4 (15%)	8 (16%)	5 (11%)	9 (17%)	3 (12%)	6 (38%)	6 (30%)	9 (43%)	4 (20%)	192 (64%)
1 very small lesion.	8 (9%)	6 (22%)	3 (6%)	2 (4%)	4 (7%)	1 (4%)	1 (6%)	2 (10%)	2 (10%)	2 (10%)	30 (10%)
2 or 3 very small lesions.........	8 (9%)	1 (4%)	9 (18%)	4 (9%)	3 (6%)	7 (29%)	2 (12%)	....	4 (19%)	1 (5%)	22 (7%)
Few small or larger lesions.........	18 (20%)	3 (11%)	4 (8%)	10 (23%)	11 (21%)	4 (17%)	3 (19%)	5 (25%)	3 (14%)	5 (25%)	27 (9%)
Multiple lesions...	38 (47%)	13 (48%)	27 (52%)	24 (53%)	26 (49%)	9 (38%)	4 (25%)	7 (35%)	3 (14%)	8 (40%)	26 (9%)
Typical lesions (included among multiple lesions).	14 (1 %	7 (26%)	14 (28%)	9 (21%)	10 (19%)	2 (8%)	....	2 (10%)	1 (5%)	1 (5%)	4 (1⅓%)

liver in control animals and in animals subjected to various experimental procedures, this conclusion of Mallory's appears to be justified.

In order to decide definitely how far the necrosis which we found in the livers of guinea pigs was caused by our experimental procedures, and how far it was apparently due to other spontaneous factors not analyzed, it was necessary to determine first the frequency and extent of necroses of the liver in a large number of control animals.

For this purpose we examined the livers of 297 guinea pigs, some of which served also as controls for the animals used for intravenous injections of various substances. They were usually animals obtained from the same breeder and at the same time as the animals which were injected. We added as controls a number of animals which had been used in other experiments, especially animals in which incisions had previously been made in the uterus and ovaries.

The last columns of tables I and II show the frequency and extent of the necroses in the liver of these control animals.

TABLE II.

	Group A.	Group B.	Controls.
No. of animals	261	101	297
No lesion	41 (16%)	28 (28%)	192 (64%)
1 very small lesion	23 (8%)	8 (8%)	30 (10%)
2 or 3 very small lesions	25 (10%)	14 (13%)	22 (7%)
Few small or large lesions	46 (17%)	20 (20%)	27 (9%)
Multiple lesions	128 (49%)	31 (31%)	26 (9%)
Typical lesions (included among multiple lesions)	54 (21%)	6 (6%)	4 (1⅓%)

We see that 64 per cent. of the control guinea pigs were free from lesions, while 17 per cent. of the remaining animals had only very slight necrosis. 9 per cent. had a few small or larger necrotic areas, and only 9 per cent. had marked multiple lesions. Of these latter animals 1⅓ per cent. had the typical multiple necrosis in which certain more or less extensive parts of the livers were dotted with small necrotic spots between which at some places larger conglomerate necrotic areas could be found.

If we analyze further the occurrence of necrosis in the liver of

the control animals, all but one of the cases in which multiple lesions were found occurred in animals which had been examined either within five days following an operation (usually affecting the ovaries or uterus), or one or two days after arrival from a railway journey lasting more than twenty-four hours, and during the hot summer months. In these two classes of control animals exclusively the severe (typical) lesions were found. Many control animals were examined twelve to fifteen days after an operation; these were in most cases free from macroscopical lesions of the liver (table III).

TABLE III.

	Total.	Examination within 5 dys. of operation.	Sick preceding death.	Day after arrival on which examined.	Bled.
No lesion	4	2	1	1	–
1 very small lesion	6	5	–	–	1
2 or 3 very small lesions	4	1	1	2	–
Few small or larger lesions	8	3	1	4	–
Multiple lesions	17	7	1	9	–
Typical lesions (included among multiple lesions)	4	1	–	3	–

If we compare with these control animals, guinea pigs which received, usually twenty to twenty-eight hours before examination, an intravenous injection of a solution of casein, nucleoproteid, serum globulin, hirudin, or of colloidal copper, we find very much more marked necrosis. The difference between these groups and the control animals becomes especially clear if we compare the frequency of severe lesions in both classes of animals. In table II under the heading of group A, the average frequency of 261 animals is given. Marked multiple lesions were observed in almost one half of the animals, and in not quite one half of the animals showing multiple lesions the liver showed over certain areas the typical dotted appearance. On the other hand, in animals injected with distilled water, solutions of egg albumin, protamin, starch, and strophanthin (group B), the lesions, although much more marked than in control animals, were distinctly less than in those animals united under group A. In group B especially the severe lesions are less marked than in group A. Altogether we examined 659 guinea pigs. Considering the large number of the animals examined our

results are sufficiently concordant to present the following conclusions: (1) In a certain percentage of guinea pigs there occurs apparently spontaneous necrosis of the liver. This necrosis is very much more severe and frequent in animals subjected to injurious influences, as to a long journey in hot weather, or to an abdominal operation. Within a few weeks after the exposure to the injurious influence, the majority of the necroses seem to disappear. In healthy animals not subject to these injurious influences, necrosis of the liver is on the whole not frequent, although small lesions may occasionally occur. (2) Intravenous injections of a great variety of substances dissolved in water, and even of distilled water cause a marked increase in the necrotic lesions found in guinea pigs. It seems, however, that different substances show an unequal tendency to produce these lesions.

Microscopical Examination of the Lesions.—Most lesions were examined approximately one day after the injection of the various substances. Some, however, were examined at an earlier period; others, from six to ten days after the injection. Many lesions in control animals were also examined. We found the lesions in the beginning situated somewhere between the portal and central areas. If the lesions were larger they sometimes extended to the portal or central veins. At first the lesions were isolated and well defined. If they became more extensive neighboring lesions could join and form conglomerate lesions. Small lesions consisted of a single cell or of a few cells with somewhat shrunken, dense nuclei and hyaline cytoplasm which stained markedly with eosin. In larger lesions and at a later period, at first the center and later the greater part of the necrotic area showed signs of autolysis, the nuclei disappeared, the cytoplasm became vacuolar, and in the end a network of fibers remained. Very soon polynuclear leucocytes collected in the necrotic areas. At first they were situated in the capillaries, later they penetrated into the cells and helped to dissolve them. There were also visible occasionally some cells which probably represented swollen endothelial cells of the capillaries of the liver or a few isolated immigrated connective tissue cells. Thrombi occluding the vessels were absent, if we do not regard collections of polynuclear leucocytes in the capillaries, the number of which, however, varied

exceedingly at different places and in different specimens, as representing cell thrombi. It is probable that the collection of the polynuclear leucocytes is a secondary phenomenon, the necrosis of some liver cells being the primary change which caused the leucocytes to collect around the diseased cells. Connective tissue cells begin to grow into the necrotic areas, and in pieces taken out six days after the injection the nuclei in the necrotic areas have faded; connective tissue has supplanted a considerable part of the material. The ingrowing connective tissue cells may assume the shape of epithelioid cells. Proliferating bile ducts may be visible in the growing connective tissue. Ten days after the injection areas of newly formed connective tissue with dilated blood vessels and proliferated bile ducts and with some polynuclear leucocytes are visible. The necrotic material has entirely disappeared at that period. Hemorrhages are frequently visible in and around the necrotic areas; even ten days after the injection there may still be present some signs of hemorrhage. They are perhaps especially marked after injection of hirudin. At other times, however, hemorrhages may be absent.

We may conclude from the microscopical studies (1) that thrombi are not responsible for the origin of the necrosis, (2) that the lesions have usually an intermediate position, and (3) that they become organized through connective tissue.

Notwithstanding their substitution by connective tissue and the relative frequency of these lesions, they evidently do not lead to cirrhosis of the liver. Cirrhotic changes in the liver of the guinea pig must be extremely rare. It seems that after a few weeks these lesions disappear entirely or almost entirely, without causing retractions at the surface of the liver, notwithstanding the fact that they are situated preferably near the surface of the liver. In our investigations into experimental myocarditis we also had indications of the apparent gradual disappearance of the lesions. For the production of cirrhosis there must perhaps occur extensive and repeated necrotic changes in a certain area.

The fact that so many different conditions have the same effect, causing focal necrosis of the liver, suggested the possibility that one common factor may be present in all of them. The idea suggested itself that the presence of bacteria in the circulation and their

subsequent retention in the liver might be responsible for the lesions. We searched, therefore, a certain number of animals showing necrosis of the liver for the presence of bacteria either in the blood or in the liver. We examined three control guinea pigs which showed necrotic spots in the liver. A platinum wire was pushed into the necrotic areas, withdrawn, and the adhering material distributed into bouillon tubes. In one case blood was withdrawn from the heart and mixed with sterile bouillon. The results were negative. In four animals in which through intravenous injection of colloidal copper necrosis of the liver had been produced, a similar bacteriological examination of the necrotic areas also proved negative. The colloidal solution of copper used for injection was found to be sterile. The results obtained after injection of a solution of hirudin were, however, positive. In five animals we examined the heart blood bacteriologically approximately one day after the injection of the hirudin, and we found bacteria in one animal. In nine animals we examined the necrotic spots in the liver for bacteria by means of cultural methods, and found them present in four animals. An examination of the hirudin solution used for injection showed the presence of apparently similar organisms to those found in the blood or necrotic spots of the liver. It is probable that in the case of hirudin bacteria were introduced into the circulation of the animals with the solution and that the bacteria afterwards lodged in the necrotic areas. We may, however, on the basis of our negative results in control animals and in animals injected with colloidal copper, conclude that in general bacteria are not responsible for the liver necrosis produced through the various experimental interferences.

An analysis of the shape and distribution of the necrotic area as it reveals itself to macroscopic examination aids us somewhat in the etiologic interpretation of these lesions. The edges and the superficial parts of the lobes of the liver are favored,—an observation made also by previous investigators in the case of other necroses of the liver. At these places the circulation is most easily interfered with, and interference with the circulation may, therefore, be suggested as one of the factors of importance in the causation of this necrosis. Frequently the necrosis has the shape of straight lines, sometimes

running almost parallel to the suspensory ligament of the liver. Occasionally they seem to be located at places where the ribs exert pressure on the liver. It is not uncommon for a lesion to be continued from one lobe to an adjoining one, as if the two lobes formed one connected whole. This is in some cases probably due to the fact that pressure is simultaneously exerted on two overlapping or adjoining lobes; in other cases, where bacteria cause the lesions, it may be due to a transmission of the organisms from one lobe to the neighboring one through contact.

The readiness with which this necrosis is produced seems to vary greatly in different species of animals. While in the guinea pig it is evidently easy to produce necrosis of the liver, it is much more difficult to do so in the mouse. In the mouse we did not observe necrosis produced by one injection of either colloidal copper or hirudin, as in the guinea pig. In one mouse we found necrotic spots in the liver after repeated injections of hirudin.

At present it is not possible to give a definite analysis of the factors causing necrosis in the liver of guinea pigs. Some are in all probability caused by certain bacteria lodging in the liver. But this explanation cannot apply to the majority of the necroses produced in our experiments. The various methods which we use have in all probability one or two factors in common, and in every case these cause the necrosis. We have indications that mechanical factors that weaken the circulation at certain places in the liver are at least partly responsible for the origin of the necrosis. It may be that injection of the various substances contributes to the production of the necrosis by reducing still further the pressure with which the circulation is maintained in the liver, or that these substances reduce the resistance of the liver cells to unfavorable conditions of the circulation by altering the metabolism of the liver cells. We are tempted to analogize the necrosis of the liver produced experimentally through these injections with the ulcer of the stomach produced by Rehfuss[3] in the guinea pig through the injection of venom of Heloderma and of various other substances. All that these substances have in common is a marked influence on the general circulation and vitality of the animal. It is probable that

[3] Rehfuss, M. E., *Carnegie Institution of Washington Publications,* 1913, No. 177, 125.

as a result of the weakening of the circulation the epithelial cells of the gastric mucosa become injured, lose their power of resistance to the action of the gastric juice, and are, therefore, digested by the latter. In both cases thrombi are not the primary cause of the lesions; hemorrhages are common to both lesions; in both cases very diverse substances can produce the same result and the common factor underlying the action of all of them is probably a reduction of the vitality of the cells either through lowering the blood pressure or through toxic interference with the metabolism of the cells. In both cases secondary factors are added,—mechanical factors (pressure) in one case, digestive action of the gastric juice in the other. We may add that the intravenous injection of hirudin causes in a certain number of cases not only necrosis of the liver but also gastric ulcers. Hemorrhage is probably the cause of these ulcers.

Necrosis of the liver has been produced experimentally in the liver of animals (Flexner,[4] Theobald Smith,[5] Boxmeyer,[6] Pearce,[7] Joannovics,[8] and Opie[9]). The necrosis observed by Flexner after injection of ricin showed much similarity to the necrosis observed by us in the liver of the guinea pig. Flexner found no definite relation between the necrosis and thrombi in the blood vessels supplying the necrotic area. He therefore doubts an etiological significance of thrombi which may occasionally be found in the affected areas; he thinks it possible, however, that lesions in the capillaries lead to an increased transudation of toxin-containing lymph and may thus be responsible for the focal necrosis in the liver. Pearce laid special stress on the occurrence of agglutinative thrombi in the vessels of the liver accompanying necrosis produced through intravenous injection of hemolytic serum, and he referred the necrosis in the main to interference with the circulation caused by these thrombi, although he admitted the possibility of other factors playing a part

[4] Flexner, S., *Johns Hopkins Hosp. Rep.*, 1897, vi, 359; *Jour. Med. Research*, 1902, viii, 316; *Med. News*, 1894, lxv, 116.

[5] Smith, T., and Moore, V. A., *U. S. Department of Agriculture, Bureau of Animal Industry*, 1894, *Bulletin No.* 6.

[6] Boxmeyer, C. H., *Jour. Med. Research*, 1903, ix, 146.

[7] Pearce, R. M., *Jour. Exper. Med.*, 1906, viii, 64.

[8] Joannovics, G., *Ztschr. f. Heilk.*, 1904, xxv, 25.

[9] Opie, E. L., *Jour. Exper. Med.*, 1910, xii, 367.

in their origin. One of us has shown[10] that the necrosis produced through intravenous injection of ether is due to two factors: (1) a direct injurious action of the ether on liver tissue, and (2) autolytic changes induced through clotting in the larger blood vessels. The presence of the first factor could be demonstrated in an exact manner through observation of the development of the necrosis *in vitro* under conditions where interference with the circulation no longer could determine the origin of localized, focal necrosis at the same places where the necrotic areas would have arisen in the living body. At a later date the extension of the necrosis took place in the area supplied by blood vessels thrombosed as a result of the primary action of the ether and of the primary necrosis. The clotted blood vessels at this period are no longer open to the passage of carmin particles injected into the vessels of the liver. Karsner and Aub[11] were not able to produce, through injection of hemolytic serum, the same effects as those obtained by us through injection of ether. This was not to be expected inasmuch as hemolytic serum acts very much less strongly than does ether introduced into the circulation. Karsner and Aub were, therefore, unable to determine the manner in which the injection of hemolytic serum produces necrosis in the liver. It may be suggested that possibly some light may be thrown on the relative significance of direct toxic action on the blood vessels and liver tissue and of secondary effects due to thromboses by an exact determination of the time relations in the development of the necrosis. It is to be expected that effects due to a direct toxic action appear earlier than the effects due to thrombosis, the latter proceeding approximately at the same rate as the autolytic processes due to complete ligation of a lobe of the liver.

SUMMARY.

Through the intravenous injection of various substances differing very much in character, multiple necrosis can be produced in the liver of the guinea pig. In the mouse the effect of these substances is absent or much less marked. Different substances seem to differ,

[10] Loeb, L., and Meyers, M. K., *Virchows Arch. f. path. Anat.*, 1910, cci, 78.

[11] Karsner, H. T., and Aub, J. C., *Jour. Med. Research*, 1913, xxviii, 377.

however, in their power to produce necrosis. In control animals necrosis in the liver is much more rare. It is found especially in animals subjected to various injurious influences. The necrotic areas are usually situated between the portal and central areas of the liver acini. Their development is not due to thromboses interfering with the circulation in certain areas of the liver. They are probably due to a weakening of the circulation in the liver or to interference with the metabolism of the cells as a result of the injection of foreign substances. Mechanical factors (pressure on the liver cells) may have an additional effect. This necrosis may be compared etiologically to the acute gastric ulcers which can be produced through a great variety of toxic substances in the guinea pig.

THE EFFECT OF THE INTRAVENOUS INJECTION OF SUBSTANCES AFFECTING TUMOR GROWTH ON THE CYCLIC CHANGES IN THE OVARIES AND ON PLACENTOMATA.*

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In preceding communications[1] we described the effect of the intravenous injection of various substances on tumor growth and on the liver of guinea pigs. In the present report we shall compare with the data obtained in these studies, the effect of the intravenous injection of some of these substances on experimentally produced placentomata, which represent rapidly growing tumor-like formations, and on the cyclic changes of the ovaries.

THE EFFECT OF THE INTRAVENOUS INJECTION OF HIRUDIN ON THE OVARIES.

In four experiments hirudin was injected intravenously[2] into female guinea pigs on the ninth or tenth day of the sexual cycle, and the animals died within a short time of the injection (four and one half to seven and one half hours after the injection; in one case the exact time of death was unknown, but the animal died within the first twenty-four hours). Six to seven days after ovulation, incisions had been made, under ether anesthesia, in the uterus. In these cases no marked changes were found in the ovaries, although, as we shall see later, a decided effect had been produced on the placentomata. There was perhaps a slightly increased degeneration of the large follicles, but it was not significant. There was in some cases an increase in the number of polynuclear leucocytes in the corpora

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[1] Fleisher, M. S., and Loeb, L., *Jour. Exper. Med.*, 1914, xx, 169. Loeb L., and Fleisher, M. S., *Jour. Am. Med. Assn.*, 1913, lx, 1857.

[2] 4 to 8 c.c. of a 0.25 per cent. solution of hirudin were injected each time.

lutea, mainly in the capillaries, but in some cases the leucocytes had entered the lutein cells.

In two cases the ovaries were examined one day after the injection of hirudin. In one of the cases the large and medium sized follicles and some of the small follicles showed degeneration of the granulosa. A mature follicle was, however, well preserved. In the corpus luteum there was some vacuolization in the periphery. In this case incisions had been made into the uterus six and one half days after ovulation, and three days later the hirudin was injected. In the other case hirudin was injected intravenously in a pregnant guinea pig. In this case we found also in all the large and medium follicles degeneration of the granulosa, while the mature follicles, as well as the small follicles, were well preserved. The capillaries of the corpora lutea were crowded with polynuclear leucocytes to such an extent that some of the capillaries appeared occluded; some lutein cells were necrotic. In a third case, in which without previous incisions into the uterus, hirudin had been injected six and one half days after copulation, the ovaries were normal.

We may conclude that within the first twenty-four hours after injection of hirudin the degeneration of the granulosa sets in at first in all the large and medium sized follicles and subsequently also in some small follicles. This degeneration of the granulosa is followed by ingrowth of connective tissue and complete atresia of the follicles. The mature follicles are considerably more resistant than the other large follicles,[3] and in a similar manner as they survive the injurious effect of those conditions which lead to ovulation, while the other follicles perish at that time, they may also survive the effect of hirudin, to which the other follicles succumb. We may find a large number of polynuclear leucocytes in the corpora lutea and also some degeneration of the lutein cells. At the same time we see that hirudin does not in all cases have this effect on the ovaries.

If about seven days after copulation incisions are made, under ether anesthesia, into the uterus, three days later hirudin is injected, and two or three days after the injection the ovaries are examined, we find only good follicles of small or small to medium size, while

[3] Loeb, L., *Centralbl. f. Physiol.*, 1911–12, xxv, 336; *Jour. Morphol.*, 1911, xxii, 37.

the large majority of follicles are found to be in various stages of connective tissue atresia. There may still be found some large follicles undergoing degeneration of the granulosa. In the corpora lutea we may or may not find collections of polynuclear leucocytes and some vacuolization and karyorrhexis in the lutein cells. In another case, however, in which nine days after copulation the hirudin had been injected without previous incision into the uterus, no changes were found in the ovaries.

In a further series of experiments two or three injections of hirudin were given in succession. In the majority of cases the incisions into the uterus were made six to seven days after copulation; three and five days following the operation hirudin was injected. The ovaries were examined from a few hours to three days after the last injection. In all these cases we find approximately the same picture in the ovaries; namely, good follicles of small or small to medium size and follicles in various stages of connective tissue atresia. There are especially many follicles in the last stage of connective tissue atresia; in one case some large follicles with degenerating granulosa were still present. In several other cases mature follicles were seen in which degeneration of the granulosa had set in. As we mentioned previously, mature follicles are very resistant, but they also degenerated under the influence of several injections of hirudin. In the corpora lutea we often found dilated vessels; polynuclear leucocytes might be present or absent. Some vacuolization of the lutein cells could sometimes be observed in the corpora lutea of these animals; it may, however, begin to set in normally at this period of the sexual cycle. The same changes were found in cases in which three instead of two injections of hirudin had been given.

In two cases of pregnancy where, without previous incisions into the uterus, two injections of hirudin were given, the changes were not as definite in the ovaries as in the previous experiments. In one of these two pregnant animals abortion followed the injection of hirudin.

From these experiments we may conclude that if six to seven days after copulation incisions are made into the uterus under ether anesthesia, and on the following days one or several injections of

hirudin are given, changes set in in the ovaries which correspond to those found at the time of ovulation; namely, a degeneration of all with the exception of the small follicles. These degenerative changes are followed by the same developmental changes which one of us has described previously in the ovaries.[4] In the cases that were observed for a certain period of time following the injection, the ovaries gradually resumed their normal function and ovulation occurred again. The corpora lutea are also sometimes, but not always, affected as a result of these injections. We found thus a second method of altering experimentally the periodicity of the sexual cycle; the first one consisted in the early extirpation of the corpora lutea, which one of us has described previously.[5] In several cases in which the injection of hirudin was given without previous incisions into the uterus, the ovaries were not at all, or were only slightly affected.

THE EFFECT OF THE INTRAVENOUS INJECTION OF HIRUDIN ON THE PLACENTOMATA.

In the majority of the guinea pigs referred to in the previous paragraph incisions had been made into the uterus of the anesthetized animals approximately five and one half to seven days after copulation. This operation was followed within the next week by one or several intravenous injections of hirudin. As a result of the incisions into the uterine wall placentomata began to develop within a few days after the operation. We were, therefore, in these experiments, in a position to determine the effect of hirudin on the placentomata.

In four animals in which the hirudin was injected about three days after the operation and in which the placentomata were examined, in three cases four and one half to seven hours, and in one case within the next twenty-four hours following the injection, as a result of the injection of hirudin by far the greater part of the developing placentomata had been destroyed by hemorrhage; however, small placentomatous areas near the mucosa had been pre-

[4] Loeb, L., *Jour. Morphol., loc. cit.; Virchows Arch. f. path. Anat.*, 1911, ccvi, 278.

[5] Loeb, L., *Deutsch. med. Wchnschr.*, 1911, xxxvii, 17.

served; in the preserved areas there may have been some hyperemia; the number of mitoses in the cells of the placentomata was either very small or mitoses were entirely missing. The adjoining mucosa of the uterus was occasionally found to be edematous.

In two animals in which the placentomata were examined one day after the injection of hirudin, necrosis had set in in the greater part of the placentomatous tissue as a result of interference with the nourishment following the hemorrhages. In the majority of animals the placentomata were examined at a later period, about six to seven days after the operation, after one or several injections of hirudin had been given and several days after the last injection of hirudin. In these cases we noticed on macroscopical examination that the placentomata were much reduced in size; in some cases it was difficult to decide whether placentomatous tissue developed at all. Microscopically we find most of the placentomatous tissue destroyed by hemorrhage and necrotic. There were usually present some small islands of living placentomatous tissue which were situated near the mucosa, at places where the circulation had been less interfered with. But even in these areas, as well as in the adjoining mucosa, there were present indications of passive congestion. In the living placentomatous tissue mitoses were either missing or very few in number. The unfavorable circulatory conditions prevented probably a very active cell proliferation under those conditions. Hemorrhages could occasionally also be seen in the adjoining mucosa. In some cases collections of polynuclear leucocytes were present in the necrotic areas. They were in part attracted by the substances given off by the necrotic tissue. It is, however, probable that at least occasionally bacterial infection played a part. We found in some cases in which we examined the animals after injection of hirudin that certain bacteria developed at the place of injection; and even in the heart blood and in necrotic areas of the liver produced through these injections, bacteria could be discovered after intravenous administration of hirudin, while after injection of colloidal copper bacteria could not be found under those conditions. It is therefore possible that bacteria were retained in the necrotic area of the placentomata, and formed an additional attraction for the leucocytes. We observed one case in which hirudin failed to exert its usual effect on the placentomata.

In four cases the hirudin was once or twice injected at a certain stage after copulation without preceding incisions into the uterus. In two of these cases, early cases of pregnancy about ten to thirteen days after copulation, no abnormality could be found in the pregnant uteri; in two other cases in which the pregnancies were somewhat further advanced, hemorrhage took place in the neighborhood of the placenta, as a result of the injections of hirudin, with the subsequent death of the embryo in one case. We may assume that in both cases abortion would have taken place as a result of the injection, if the guinea pigs had been permitted to live for a longer period after injection of hirudin.

We may then conclude that intravenous injections of hirudin destroy in the majority of cases the greater part of the placentomata through hemorrhage, and they prevent the remaining placentomatous areas from active proliferation, probably also as a result of interference with the circulation. They may cause abortion in pregnant animals. While in the case of the placentomata hemorrhages are mainly responsible for the destructive action of hirudin, the action of hirudin on the ovarian cycle must have a different cause. Hemorrhages are missing in the ovaries. It appears probable that interference with the nourishment of the follicles as a result of the action of hirudin on the blood, or on the exchange of substances between blood vessels and tissue cells, is responsible for this effect.

THE RELATION BETWEEN INCOAGULABILITY OF THE BLOOD AND A TENDENCY TO HEMORRHAGE.

Our observations on the effect of hirudin on the placentomata suggest some general considerations on the possible relation between a tendency to hemorrhage and a decrease in the coagulability of the blood. In cholemia, in hemophilia, and in purpura such a connection exists. Morawitz and Bierich[6] found that if the blood of a dog is made free of fibrinogen and the blood coagulation is retarded, a hemorrhagic diathesis is not observed. These authors come therefore to the conclusion that there exists no direct connection between

[6] Morawitz, P., and Bierich, R., *Arch. f. exper. Path. u. Pharmakol.*, 1907, lvi, 115.

the retardation of the coagulation of the blood and the tendency to hemorrhage.

In our observation we find another instance of a parallelism in the retardation of the coagulation of the blood and in the tendency to cause hemorrhages in the case of hirudin. Intravenous injection of hirudin almost invariably causes hemorrhages into the placentomata. It frequently causes hemorrhages in mouse tumors after injection into the vein of the tail of the mouse. We stated in a previous paper[7] that hirudin frequently leads to necrosis in the liver of guinea pigs, and that in the neighborhood of this necrosis, hemorrhages may be found. We did not make a systematic study of other changes produced in the body through intravenous injections of hirudin, but we noticed in several cases erosions or ulcers and some hemorrhages in the wall of the stomach of guinea pigs, and in another case we found hemorrhage in the kidney tissue, blood casts and hyaline casts in the tubules, and some hyperemia of the glomeruli of the kidney. We may therefore conclude that injection of hirudin produces a tendency to hemorrhage in various parts of the body, and that the hemorrhages are prone to occur especially at such places where the blood vessels are not well formed, or are less resistant, as in tumors and in placentomata, or in the neighborhood of necrotic areas; and that in the stomach hemorrhages may be followed by digestion of the tissue through the gastric juice. It appears probable that there is a connection between the action of hirudin on the blood (effect on the coagulability or on the viscosity) and the tendency to hemorrhage which we noticed.

THE EFFECT OF VARIOUS OTHER SUBSTANCES ON THE OVARIES AND ON PLACENTOMATA.

A number of control experiments were carried out. We found changes in the ovaries after injection of hirudin especially in animals in which placentomata had been produced and in which hemorrhages into the placentomata had taken place. It was therefore conceivable that loss of blood in itself might produce some changes in the ovaries.

In each of three guinea pigs five cubic centimeters of blood were

[7] Fleisher and Loeb, *loc. cit.*

withdrawn from the jugular vein twice, an interval of two days elapsing between the first and second bleeding. On examination three days after the last bleeding, the ovaries and uterus were found to be normal in two animals; in the third animal the ovaries were hypotypical.[8] There were small and small to medium sized good follicles, many follicles in early and medium connective tissue atresia, there were many contracted follicles, and yellow scar-like bodies, the remains of former corpora lutea. The uterus showed low cuboidal epithelium in surface and glands; there were a few mitoses in the surface epithelium. The connective tissue of the mucosa was fibrillar and contained small nuclei. Some small mononuclear cells migrated from the connective tissue into the surface epithelium. It is not probable that the bleeding is responsible for the hypotypical changes in the ovaries of one of these animals and we may conclude that withdrawal of blood is without marked effect on ovaries and uterus.

In four anesthetized guinea pigs in which incisions into the uterus had been made about seven days after copulation, ten cubic centimeters of distilled water were injected intravenously two to nine days later. The examination was made one to four days following the injection. In three cases ovaries and placentomata were normal; in one case in which the animal was not in good condition one day after the injection, degeneration of the granulosa occurred perhaps somewhat earlier than usual, in some follicles of medium size. In a rabbit in which also placentomata had been produced experimentally, intravenous injection of distilled water had no influence on the placentomata. No effect was obtained through injection of distilled water in guinea pigs in which no incisions had previously been made into the uterus; one day after the injection the ovaries and uterus were found to be normal. We may conclude therefore that intravenous injection of distilled water has no distinct influence on the ovaries and uterus in the guinea pig.

In six guinea pigs the effect of repeated injections of colloidal solutions of copper on the ovaries and placentomata were tested. Six to seven days after copulation incisions into the uterus of the anes-

[8] Loeb, L., *Centralbl. f. Physiol., loc. cit.; Virchows Arch. f. path. Anat., loc. cit.*

thetized animals were made, and in four cases four, in one case three, and in the last case two injections of colloidal copper were given. Ten cubic centimeters of the solution were injected each time. The first injection was given three or four days after the uterine operation; the following injections were made on the following days, and the organs were usually taken out for examination one day after the last injection. The ovaries were found to be normal in all cases examined; in one case, however, all the large follicles showed degeneration of the granulosa. It is doubtful whether this was due to the influence of the colloidal copper. The placentomata in these cases were much larger than in the animals which had received hirudin; their size was approximately normal. There was, however, in most cases some hemorrhage and necrosis in the placentomata and it is possible that the injections of colloidal copper increased the hemorrhage and necrosis in the placentomata, which to some extent may be found even in guinea pigs that had not received any injection. There was, however, usually a considerable amount of living placentomatous tissue present with numerous mitoses. In two rabbits which had been similarly treated good deciduomata were found, and in one case a normal pregnancy was present.

We may conclude that repeated intravenous injections of colloidal copper are without effect on the ovaries of guinea pigs and without marked effects on the placentomata of guinea pigs and rabbits. They may possibly increase somewhat the amount of hemorrhage and necrosis, often to some extent normally present in further developed placentomata.

In twelve guinea pigs either one or two intravenous injections of nucleoproteid prepared from the kidney or lymph glands of cattle were given after incisions into the uterus of the anesthetized animals had been made about six to seven days after copulation. The first injection was given two to five days (in one case nine) after the operation; the second injection one to two days after the first one; two cubic centimeters of a 15 per cent. solution were given each time. Ovaries and uterus were in most cases taken out one to three days after the last injection. In the majority of cases the ovaries were found to be normal. In one case, however, one day after the injec-

tion there was much degeneration of the granulosa, even in medium sized and in some small follicles; in two other cases large follicles without degeneration of the granulosa were missing. It is therefore possible that in a few cases a slight effect on the ovaries was obtained; but in the majority of cases no effect was present. The placentomata were not noticeably influenced through the injection of nucleoproteid; occasionally some hemorrhages were observed in the placentomatous tissue.

In four guinea pigs in which six to seven days after copulation incisions into the uterus of the anesthetized animals had been made, nine to ten days after the operation intravenous injections of ten cubic centimeters of a 1⅔ per cent. solution of casein were given. The placentomata in three cases were taken out one day later; in one case the animal died ten minutes after the injection; in two cases there were extensive hemorrhages and necroses in the placentomata. In the two other cases the placentomata were in good condition. In two cases in which the ovaries were examined they were found to be normal. We have to consider the possibility that at this late stage of development of the placentomata (nine days after the operation) even mechanical factors (perhaps handling of the animals during intravenous injection) may be liable to cause some hemorrhage. In a rabbit injections of casein had no noticeable effect on the experimental placentomata or on pregnancy.

We may conclude that the various experimental interferences mentioned do not produce any marked or constant effect on the ovaries or on the placentomata.

SUMMARY.

If six to seven days after copulation incisions are made under ether anesthesia into the uterus, and on the day following this operation one or several injections of hirudin are given, changes set in in the ovaries which correspond to those found at the time of ovulation; namely, a degeneration of all the follicles with the exception of the small ones. These degenerative changes are followed by the same developmental changes as in the normal cycle. This represents a second method of altering experimentally the periodicity of the sexual cycle, the first consisting in the early extirpation of the corpora lutea described previously by one of us.

Under the same conditions intravenous injections of hirudin destroy in the large majority of cases the greater part of experimental placentomata through hemorrhages, and they prevent the remaining placentomatous areas from active proliferation, probably as a result of interference with the circulation. These injections may also cause abortion in pregnant animals.

Intravenous injections of hirudin produce a tendency to hemorrhage at various places in the body, and these hemorrhages are prone to occur, especially in rapidly growing tissues, where the blood vessels are less resistant, as in tumors and in placentomata, also in the neighborhood of necrotic areas. In the stomach the hemorrhages may be followed by digestion of the tissues through the gastric juice. It is probable that there is a connection between the action of hirudin on the blood (coagulability and viscosity) and the tendency to hemorrhage.

Withdrawal of blood, intravenous injections of distilled water, colloidal copper, nucleoproteid, or casein have no marked effect on the cyclic changes in the ovaries or on placentomata.

A STUDY OF THE CIRCULATION OF THE KIDNEYS FOLLOWING LIGATION OF ONE URETER.*

By ALBERT A. GHOREYEB, M.D.

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In this work the perfusion box was the same as that used and described in a previous paper (1). Changes had to be made in the technique of perfusion, however, as in previous experiments only one kidney, the left, had been used.

In order to perfuse both kidneys simultaneously, two methods of procedure were tried. The first consisted in the use of one large aortic and two venous cannulæ; the second in using two arterial and two venous cannulæ. The first method, as far as it was tried, proved unsatisfactory because the serum could not be made to pass through normal kidneys at the expected rate. As the vessels given off by the aorta in this region other than the renal are very numerous, and as it was thought that the delay necessary to ligate these vessels brought about changes in the renal tissues, which probably interfered with the flow of serum, the first method was ultimately discarded. The second method therefore was the one adopted. The cannulæ used in the second method were all large enough not to impede the flow. The diameters of the outlets of both arterial cannulæ were equal, the diameters of the inlets of both venous cannulæ were equal, and the diameters of the outlets of the cannulæ protruding from the side of the box were equal. The animal to be perfused was etherized and bled to death through the left femoral vessels. The abdomen was opened, and the intestines, stomach, and liver were removed. The portion of the animal between the diaphragm and the iliac crests was then taken out, the aorta and vena cava were opened, and the cannulæ placed and ligated. The serum left the serum bottle through two glass tubes entering the arterial cannulæ under the same pressure.

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For several months the work was delayed because the serum could not be made to pass as rapidly through the right kidney as it did through the left. This discrepancy was thought to be due to technique rather than to a normal difference in the kidneys. Many possible sources of error were excluded until, finally, the discrepancy was overcome by the removal of the right adrenal.

In the rabbit the left adrenal lies almost directly over the aortic attachments of the renal arteries, and it had always been removed as it made more difficult the insertion of the cannulæ into these vessels. The right adrenal lies higher up and being less in the way had been left *in situ*.

As the removal of the right adrenal allowed the serum to pass more rapidly through the right kidney, it was thought that the removal of the left adrenal had been responsible for the ease with which the serum flowed through the left kidney. It was also thought, notwithstanding all proof to the contrary, that there might be some direct relation between the adrenals and the vasoconstrictor mechanism of the kidneys. In order to explain the above phenomena, the following experiments were performed.

RABBIT 7.

The Left Adrenal Was Left in Situ and the Right One Removed.

		Drops of serum.	
	Pressure.	Right.[1]	Left.[1]
After 5 min.	60 mm.	120 +[2]	120 +
After 10 min.	50 mm.	100	100
After 15 min.	50 mm.	81	84

In rabbit 7 the ligature around the left arterial cannula had been placed between the adrenal and the kidney. As the presence of the left adrenal did not seem to interfere with the rate of flow, it was thought that perhaps this ligature might have destroyed the relation between the adrenal and kidney on this side. The following experiments were therefore performed.

[1] The figures under the words "right" and "left" represent the number of drops of serum issuing in one half minute from the cannulæ protruding from the side of the perfusion box.

[2] It was found impossible to count accurately over 120 drops in 30 seconds. In such cases, therefore, where the serum issued from the cannulæ in distinct and separate drops, yet at a rate faster than 120 per 30 seconds, the sign 120 + was used.

RABBIT 19.

	Pressure.	Drops of serum. Right.	Left.
After 5 min.	40 mm.	95	120+
After 8 min.	40 mm.	100	120+
After 10 min.	40 mm.	90	120+
Right adrenal removed.			

RABBIT 21.

	Pressure.	Drops of serum. Right.	Left.
After 3 min.	40 mm.	85	120
After 5 min.	40 mm.	80	120
After 8 min.	40 mm.	80	120+
Right adrenal removed.			
After 10 min.	40 mm.	120	120+
After 15 min.	40 mm.	120+	120+

In these rabbits the ligatures around the arterial cannulæ were placed between the adrenals and aorta. During the first part of the experiment both adrenals were left *in situ*. During the latter part, the right adrenal was removed. In rabbit 19 air entered the right arterial cannula after the removal of the adrenal and the latter part of this experiment was consequently discarded.[3]

Having found a method whereby the serum could be made to flow simultaneously through both kidneys with the same pressure, at the same rate, two normal rabbits, each of which weighed between 1,500 and 2,000 grams, were perfused. The following results were obtained.

RABBIT 3.

	Pressure.	Drops of serum. Right.	Left.
After 5 min.	60 mm.	100	100
After 10 min.	50 mm.	103	100
After 15 min.	50 mm.	100	100

RABBIT 20.

	Pressure.	Drops of serum. Right.	Left.
After 5 min.	40 mm.	120	120
After 10 min.	40 mm.	120	120
After 15 min.	40 mm.	120	120

The flow through the normal kidneys in this series is apparently more rapid than that obtained previously (1), but this is explained by the small outlets to the cannulæ protruding from the side of the perfusion box, and the consequently smaller and more frequent drops.

In order to exclude nephropathic kidneys, only those rabbits were used the urine of which was free from albumen. Despite this, many of the rabbits on being opened were found to have diseased kidneys. In most of these rabbits the operation was continued and a ureter was tied as it was thought that the changes in nephropathic kidneys might be different from those in the normal kidney.

[3] Further attempts will be made to determine the exact relation between the adrenal gland and kidney perfusion.

In order to ligate the ureter the rabbits were etherized, the abdomen was opened, either the right or left ureter was ligated with silk, and the abdomen was then closed. Complete aseptic precautions were observed throughout. Several of the animals operated on died a few days after operation. The cause of death in these animals was not ascertained (2). It may be said, however, that the majority of the rabbits that died were from those that had changes in the kidney prior to ligation of the ureter.

With the object in view of finding out whether the intrapelvic pressure brought about by ligation of the ureter impeded circulation through the kidney (3) and whether this impediment gave rise to a compensatory increase in the flow through the other kidney, the first two rabbits operated on that were found to be normal were allowed to live three weeks before being perfused. Of these two animals, the hydronephrotic kidney of rabbit 1 was found to have become infected and consequently the changes produced therein by pressure could not be determined. The opposite kidney, however, other than being decidedly hypertrophied, showed no infection. Therefore, the animal was perfused with an idea of determining whether the flow through the hypertrophied kidney had materially increased. Rabbit 3 showed no infection.

RABBIT 1.

Right Ureter Ligated.

	Pressure.	Drops of serum. Right.	Left.
After 5 min.	40 mm.	5	Stream.[4]
After 10 min.	40 mm.	5	Stream.

RABBIT 3.

Left Ureter Ligated.

	Pressure.	Drops of serum. Right.	Left.
After 3 min.	40 mm.	Stream	0
After 5 min.	120 mm.	Pressure shut off	60
After 7 min.	60 mm.	Stream	14
After 10 min.	60 mm.	Shut off	16
After 14 min.	60 mm.	Shut off	14
	Sac punctured.		
After 14 min.	60 mm.	Stream	40
After 20 min.	60 mm.	Stream	48
After 25 min.	60 mm.	Stream	48

The conclusions drawn from the above experiments are as follows: (1) The intrapelvic pressure produced by ligation of a ureter impedes circulation through the kidney. (2) At the end of three

[4] Serum drops of approximately over 175 per minute lost their identity and merging formed a stream. In such cases the word "stream" is used.

weeks there is a compensatory increase in the rate of flow through the vessels of the opposite kidney. (3) At the end of three weeks after ligation of the ureter, changes occur in the kidney tissue which in themselves are sufficient to impede circulation.

On arriving at the above conclusions, the following experiments were performed with the object in view of determining how soon after ligation of the ureter the changes in the kidney tissue became sufficiently developed to produce an impediment to the flow. In this series the perfusion of the rabbits was begun as heretofore. When the flow through both kidneys had reached its maximum rate, the hydronephrotic sac was punctured and the perfusion then continued until the flow through the damaged kidney had again reached its maximum.

RABBIT 15.

Perfused Twenty-Four Hours after Ligation of Left Ureter.

	Pressure.	Drops of serum. Right.	Left.
After 5 min.	60 mm.	120 +	9
After 8 min.	60 mm.	120 +	9
	Sac punctured.		
After 10 min.	60 mm.	120 +	10
After 15 min.	60 mm.	120 +	10

For lack of serum the experiment was here discontinued.

RABBIT 12.

Perfused Forty-Eight Hours after Ligation of Left Ureter.

	Pressure.	Drops of serum. Right.	Left.
After 5 min.	60 mm.	120 +	2
After 10 min.	60 mm.	120 +	2
	Sac punctured.		
After 13 min.	60 mm.	120 +	9
After 18 min.	60 mm.	120 +	78
After 20 min.	60 mm.	120 +	112
After 25 min.	60 mm.	120 +	120 +

RABBIT 13.

Perfused Forty-Eight Hours after Ligation of Right Ureter.

	Pressure.	Drops of serum. Right.	Left.
After 5 min.	40 mm.	9	120 +
	Sac punctured.		
After 10 min.	50 mm.	90	120 +
After 15 min.	40 mm.	120 +	120 +

RABBIT 14.

Perfused Seventy-Two Hours after Ligation of Left Ureter.

	Pressure.	Drops of serum. Right.	Left.
After 3 min.	60 mm.	Stream	13
After 5 min.	40 mm.	Stream	10
After 8 min.	60 mm.	Stream	28
After 10 min.	60 mm.	Stream	42
After 12 min.	40 mm.	Stream	70
After 15 min.	40 mm.	Stream	70
	Sac punctured.		
After 20 min.	40 mm.	Stream	120

For lack of serum the experiment was here discontinued.

RABBIT 17.

Perfused Ninety-Six Hours after Ligation of Right Ureter.

	Pressure.	Drops of serum. Right.	Left.
After 2 min.	60 mm.	70	120+
After 5 min.	60 mm.	120	50
After 10 min.	50 mm.	120+	50
	Sac punctured.		
After 12 min.	50 mm.	120+	80
After 15 min.	60 mm.	120+	120

RABBIT 18.

Perfused Ninety-Six Hours after Ligation of Left Ureter.

	Pressure.	Drops of serum. Right.	Left.
After 5 min.	60 mm.	120	40
After 8 min.	40 mm.	Stream	50
After 10 min.	60 mm.	Stream	50
	Sac punctured.		
After 14 min.	60 mm.	Stream	60
After 16 min.	60 mm.	Stream	55
After 20 min.	60 mm.	Stream	50

RABBIT 16.

Perfused One Week after Ligation of Right Ureter.

	Pressure.	Drops of serum. Right.	Left.
After 2 min.	60 mm.	21	Stream.
After 5 min.	60 mm.	15	Stream.
After 6 min.	40+mm.	6	120+
	Sac punctured.		
After 10 min.	60 mm.	26	Stream.
After 12 min.	60 mm.	30	Stream.
After 14 min.	80 mm.	80	Shut off.
After 20 min.	80 mm.	82	Shut off.
After 22 min.	60 mm.	60	Shut off.
After 25 min.	60 mm.	60	Shut off.

Summary.—Increased intrapelvic pressure impedes the flow of serum through the kidney. Following ligation of the ureter a change occurs in the kidney tissues which offers an impediment to circulation other than that produced by pressure alone; this change

occurs one week after ligation. A compensatory increase in the rate of flow through the control kidney is first noticed seventy-two hours after ligation and is quite apparent at the end of one week.

HISTOLOGY.

The histological changes following ligation of the ureter have been described by many investigators (4, 5, 6). The changes will be described here in as far as they seem to be related to the problem in hand.

RABBIT 15.

Perfused twenty-four hours after operation.

Control Kidney.—No change is seen other than a slight edema associated with perfusion. A few of the glomeruli are distended with blood showing that the perfusion had not extended equally over all parts of the kidney.

Kidney with Ligated Ureter.—This kidney shows dilatation of all the convoluted and particularly the large collecting tubules near the end of the pyramids. Many of the tubules of Henle particularly the descending loops contain definite casts both hyaline and granular. Some are also found in the collecting tubules. In the cortex the primary convoluted tubules show slight swelling. Very considerable changes consisting in exudation and cell desquamation are found in the secondary tubules. None of these lesions are equally distributed; although there is general degeneration of the epithelium, the changes are more marked in single groups of tubules. The glomeruli are intact. The perfusion in this kidney is not complete, as many of the straight vessels in the pyramids contain blood. There is also much blood in the capillaries of the cortex.

RABBIT 13.

Perfused forty-eight hours after operation.

Control Kidney.—In this kidney the epithelium of the tubules in the pyramids is normal; the tubules are not dilated. There is an occasional cast in the Henle loops, and considerable edema. There is considerable edema in the tubules of the cortex with granular material in the lumen. Around all the glomeruli there is a crescent of coagulated exudation which consists in a granular mass enclosing large hyaline droplets. The perfusion here extended to all the glomeruli, the kidney being completely bloodless. This kidney is not absolutely normal, there being a few foci of cell infiltration and connective tissue increase in the cortex. The granular material in the convoluted tubules here is the same as that in the capsular spaces. The globules are usually of the same size and show a completely hyaline mass within a definite circle.

RABBIT 12.

Perfused forty-eight hours after operation.

Control Kidney.—This shows material of the same character in the capsular space, but very much smaller in amount and the hyaline globules are not so

marked. It is difficult in these cases to decide whether the material in the capsular space is due to perfusion and represents material which during the perfusion has passed through the glomeruli, or whether it had occurred during life. In many cases it is so abundant that the glomerulus is apparently compressed by it. The fact that it is not a condition common to a perfusion would seem to be evidence that it occurred during life, although it is perfectly possible that in these kidneys the glomeruli may have become more permeable.

Kidney with Ligated Ureter.—There is some dilatation of the collecting tubules, as in the previous case. Casts are present, but they are not so numerous in the tubules of the pyramids. The epithelium of the pelvic tubules shows a greater degree of degeneration, and the dilatation involves the smaller tubules of the pyramids relatively more than the larger. The dilatation involves also the tubules of the cortex, and in places even the primary collecting tubules. The secondary tubules are often dilated to double or more of the normal lumen. The epithelial degeneration in the primary convoluted tubules is well marked, and the tubules contain granular material derived from the epithelium. The most striking thing in this kidney is the greater extension of the dilatation into the tubules of the cortex.

RABBIT 14.

Perfused seventy-two hours after operation.

Control Kidney.—In this case the glomeruli are large and there is a varying amount of the same material in the capsule. Some contain a great deal, others but a small amount. In this kidney many of the glomeruli contain blood and have escaped the perfusion; elsewhere, there is much the same condition as in the kidney previously examined.

Kidney with Ligated Ureter.—The usual condition of dilatation of ducts and degeneration is seen. There is very little difference from that seen in the former kidneys.

RABBIT 16.

Perfused one week after operation.

Control Kidney.—In this kidney the exudate in the capsules is present. The epithelium generally is large and granular. There is some edema.

Kidney with Ligated Ureter.—There is the usual tubular dilatation. In the pyramids there is a distinct increase in connective tissue around the small blood vessels. There is throughout the kidney in both pyramids and cortex an increase in connective tissue. The glomeruli contain no exudate, but show an increase in the vascular cells which varies in degree.

RABBIT 3.

Perfused twenty days after operation.

Control Kidney.—The glomeruli on being measured are generally found to be larger than normally. Around most of them there is some granular material. The convoluted tubules show large, well granulated cells, and here and there in the pyramids there are foci of cellular infiltration.

Kidney with Ligated Ureter.—The dilatation of tubules here is but slightly apparent. There is a very marked increase of tissue with masses of cell infiltration in the pyramids, apparently extending from this into the cortex. The

increase of tissue in the cortex is diffuse, but accentuated in foci, and the dilatation of tubules is relatively more marked in the small collecting tubules of the cortex than elsewhere. There is marked thickening and proliferation of the epithelium of the cortex.

SUMMARY.

During the first four or five days after ligation of the ureter, the changes in the kidney are mainly degenerative in type. One week after ligation of the ureter connective tissue cells begin to appear infiltrating the kidney tissues. There is also an increase in the vascular cells of the glomeruli. These changes are well marked at the end of twenty days from the time of ligation. At the end of twenty days from the time of ligation there is also a marked hypertrophy of the control kidney, the glomeruli showing an average increase in size of twenty-five microns.

CONCLUSIONS.

During the first week following ligation of one ureter there is found an impediment to circulation which is removed upon opening the distended pelvis and reducing the intrapelvic pressure. This impediment, therefore, is probably due to the compression of the blood vessels within the kidney brought about by dilated tubules and unyielding capsule, and also possibly to disturbance of circulation produced by the distension of the pelvis. The venous and arterial branches passing over this may be either compressed or in various ways distorted. During this period the anemia produced by the impediment together with pressure and other factors gives rise to changes in kidney tissues which reveal themselves microscopically as a swelling and degeneration of the epithelial cells. Cloudy swelling was found by Karsner and Austin (7) two hours after experimental bland embolism of the renal artery.

At the end of one week following ligation of one ureter there is an impediment to the circulation other than that due to intrapelvic pressure. This impediment is probably due to cicatricial changes in the organ, which tend to maintain the tubules dilated and in a position of encroachment on the space normally allotted to blood vessels, and which manifest themselves microscopically as a connective tissue cell infiltration into the interstitial tissues of the

kidney, and an increase in the vascular cells of the tufts diminishing the caliber of the tuft capillaries.

The increased amount of work thrown upon the control kidney produces no lesions other than a hypertrophy, which permits a greater and more rapid circulation through its vascular system.

I wish to express my thanks to Dr. William T. Councilman for his aid in this study, particularly in regard to the histological examinations.

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PARAMENINGOCOCCUS AND ITS ANTISERUM.*

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In the course of the treatment of epidemic meningitis with the antimeningococcic serum it became evident that a proportion of cases of the disease treated with the serum failed to react favorably. In some of these cases the meningococcus contained in the cerebrospinal exudate survived, continued to multiply, and failed to be phagocyted by the leucocytes, in contradistinction to what happened in the majority of instances. That the meningococci occurring in the resistant cases were in part resistant, or fast, to the antiserum was suspected, and two possible kinds of fast strains were recognized: first, strains originally fast at the time the serum treatment was begun; and, second, strains developing fastness in the course of the serum treatment.[1] The second group embraced those instances in which meningococci at first reacted to the serum but later failed to do so, leading to a relapse which continued to a fatal termination.

A more precise definition of the fast strains has not thus far been made. However, Dopter[2] has studied a special class of the cocci obtained from the nasal secretion and called by him parameningococci, which while resembling the common meningococcus in fermentative and cultural properties differs from it in certain immunological reactions. The parameningococcus, so called, has now been found by Dopter and other French writers to invade the meninges and the blood and is believed by them to be one of the causes of cerebrospinal meningitis.[3] According to the French ob-

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[1] Flexner, S., *Jour. Exper. Med.*, 1913, xvii, 553.

[2] Dopter, Ch., *Compt. rend. Soc. de biol.*, 1909, lxvii, 74.

[3] The literature has been reviewed by Dr. Flexner in his article on antimeningococcic serum (Flexner, S., in Kraus, R., and Levaditi, C., Handbuch der Technik und Methodik der Immunitätsforschung, 2d edition, Jena, 1914 (in press)).

servers, when acute meningitis arises through the presence of the parameningococcus it is not controllable by means of the ordinary antimeningococcic serum, but does respond to a special antiserum prepared with cultures of the parameningococcus.

The subject of the atypical strains of the meningococcus is an important one since it affects the question of a better control of cases of epidemic meningitis by means of a serum containing antibodies for the unusual varieties. On that account we have made a study of two cultures of the parameningococcus obtained from Dr. Dopter through the kindness of Dr. Louise Pearce, who made a hurried journey to Paris to secure them, since it was found that the cultures sent by post did not survive the journey.

IMMUNITY REACTIONS OF THE MENINGOCOCCUS AND PARAMENINGOCOCCUS.

The meningococcus is subject to the several immunity reactions of agglutination, complement deviation, and opsonization. All the reactions are specific, although certain strains react not at all or imperfectly. As regards agglutination, it may be said that considerable variations arise affecting strains of meningococci relatively or absolutely inagglutinable. And yet agglutination is perhaps the most trustworthy guide in the identification of the meningococcus through specific serum reactions.[4] While immune sera may possess high agglutinating value for meningococci, normal sera possess either none, or very little; the inagglutinable strains react to immune sera of high value no more than they do to normal sera.

On the one hand, the inagglutinable strains react differently to a given meningococcic serum, while again a given agglutinable strain may react unequally to immune sera prepared from different strains of meningococci.[5] It should further be noted that while homologous sera tend to agglutinate corresponding strains best, exceptions occur in which they agglutinate heterologous strains better.[5,6]

[4] Flexner, S., in Kraus, R., and Levaditi, C., Handbuch der Technik und Methodik der Immunitätsforschung, *loc. cit.* Elser, W. J., and Huntoon, F. M., *Jour. Med. Research,* 1909, xx, 371.

[5] Eberle, J., *Arch. f. Hyg.,* 1908, lxiv, 171.

[6] St. Baecher, in Kraus, R., and Levaditi, C., Handbuch der Technik und Methodik der Immunitätsforschung, Jena, 1911, supplement 1, 80.

Notwithstanding the variations indicated, it is obviously still desirable to determine the degree in which parameningococci differ from normal meningococci in respect to immunity reactions, and to ascertain whether they form a special class or merely constitute variations from the normal type. For this purpose two cultures, L and M, of the parameningococcus secured from Dr. Dopter have been subjected to the reactions of agglutination, complement deviation, and opsonization, respecting which they have been compared with several strains of normal meningococci. The immune sera employed consisted of a polyvalent antimeningococcic serum prepared by the Department of Health of the City of New York, and several monovalent sera produced in the rabbit by immunization with single strains of the microörganisms.

It will conduce to clearness and simplicity of presentation to describe briefly the different strains of meningococci which were studied.

The parameningococci consisted of Gram-negative diplococci indistinguishable from ordinary meningococci in form, staining properties, and fermentative reactions. They were also subject to autolysis in the manner of normal meningococci.

Twenty other strains of meningococci were employed for comparison. Four, HP, MA, B, and 138, came from the Pasteur Institute, having been secured by Dr. Pearce along with the parameningococci. They were regarded as normal strains. It may be noted here that all four exhibited irregularities of agglutination, and B proved inagglutinable.

One strain, W, came from Great Britain in 1908, and was isolated from a case of posterior basic meningitis. It was a normal strain. The remaining fifteen strains were obtained in New York, partly from the stock of The Rockefeller Institute, partly from the Department of Health.[7] They have been classified as follows: Eight are normal strains agglutinating regularly. They are designated F, 20, 25, 28, 35, 45, 48, and 49. Cultures 25 and 48 were derived from rapidly fatal fulminating cases of meningitis; 28 is from a severe case terminating fatally on the fourth day; 45 is from a fatal case developing basic symptoms; 35 is from a mild case becoming chronic, in which hydrocephalus developed before death; and 49 is from a mild case which recovered.

Five are normal strains agglutinating irregularly. BH was derived from a fatal case in an infant; 9 and 18 were without history; and 37 was obtained from a case which recovered under serum treatment. Two strains, 7 and 42, gave agglutination reactions similar to the parameningococci, and had been employed in the manufacture of the antimeningococcic serum by the New York Department of Health. Table I furnishes a means of ready reference.

[7] For the Board of Health cultures I am indebted to Dr. Phoebe Du Bois and Dr. Marie Grund.

TABLE I.

Source of Cultures.

Designation.	Source.	Nature of strain.	Type of agglutination.
L	Dopter	Para	
M	Dopter	Para	
HP	Pasteur Institute	Normal	Irregular.
MA	Pasteur Institute	Normal	Irregular.
138	Pasteur Institute	Normal	Irregular.
B	Pasteur Institute	Normal	Inagglutinable.
W	Great Britain	Normal	Regular.
F	New York	Normal	Regular.
18	New York	Normal	Regular.
25	New York	Normal	Regular.
28	New York	Normal	Regular.
35	New York	Normal	Regular.
45	New York	Normal	Regular.
48	New York	Normal	Regular.
49	New York	Normal	Regular.
BH	New York	Normal	Irregular.
I	New York	Normal	Irregular.
9	New York	Normal	Irregular.
20	New York	Normal	Irregular.
37	New York	Normal	Irregular.
7	New York	Normal	Para-like.
42	New York	Normal	Para-like.

Besides the results of agglutination, other immunity reactions were studied; those, namely, of opsonization, complement deviation, and protection. These reactions are subject also to irregularities and variations, and notably that of complement deviation,[8] which has been generally given up as a method of determining the value of the antimeningococcic serum in therapeutic immunity principles.

AGGLUTINATION.

Several immune sera were employed for determining the immunity reactions. One, that of the New York Health Department, was prepared in the horse from many strains of meningococcus and preserved with 0.3 per cent. tricresol. The monovalent sera were made by immunizing rabbits with single strains. The inoculations were conducted over periods of several months. For the general

[8] Flexner, S., in Kraus, R., and Levaditi, C., Handbuch der Technik und Methodik der Immunitätsforschung, *loc. cit.*

TABLE II.

Monovalent Rabbit Serum Immune to Parameningococcus L. Agglutination Reactions Made at 55° C.

Strains.	C	10	20	50	100	200	500
Para L	–	++	++	++	++	++	+
Para M	–	++	++	+	+	+	–
48	–	+	+	±	–	–	–
45	–	+	–	–	–	–	–
49	–	++	+	±	–	–	–
42	–	++	++	++	+	+	–
35	–	++	–	–	–	–	–
37	–	+	+	+	+	–	–
138	–	+	+	±	–	–	–
28	–	++	±	–	–	–	–
25	–	++	++	+	±	–	–
20	–	++	++	++	+	±	–
18	–	++	++	++	+	±	–
9	–	++	++	+	±	–	–
7	–	++	++	+	+	–	–
B	–	–	–	–	–	–	–
MA	–	+	+	+	–	–	–
HP	–	+	+	+	–	–	–
W	–	++	+	+	±	–	–
I	–	+	+	+	–	–	–
BH	–	+	+	+	+	–	–
F	–	+	+	–	–	–	–

TABLE III.

Monovalent Rabbit Serum Immune to Parameningococcus M. Agglutination Reactions Made at 55° C.

Strains.	C	10	20	50	100	200	500
Para M	–	++	++	++	++	+	+
Para L	–	++	++	+	+	±	–
48	–	+	±	–	–	–	–
45	–	+	+	+	–	–	–
49	–	++	++	+	±	–	–
42	–	++	++	++	+	+	–
35	–	+	±	–	–	–	–
37	–	++	++	+	±	–	–
138	–	+	+	+	–	–	–
28	–	+	+	–	–	–	–
25	–	+	+	±	–	–	–
20	–	++	++	++	+	–	–
18	–	++	++	++	+	–	–
9	–	++	++	++	+	–	–
7	–	++	++	++	+	+	–
B	–	+	–	–	–	–	–
MA	–	+	+	+	+	±	–
HP	–	+	+	+	+	+	–
W	–	++	+	±	–	–	–
I	–	++	++	++	+	+	–
BH	–	+	+	+	+	+	–
F	–	+	+	±	–	–	–

work the two Dopter strains of parameningococci and normal strains 35 (mild case), 45 (basic case), and 48 (fulminating case) were used.

At the expiration of three months the titer of the rabbit sera did not exceed 1 to 500. But as normal rabbit serum is inactive in dilutions greater than 1 to 10, the specific effects could be readily followed. A few exceptions with normal strains occurred. Thus strains 45 and 138 agglutinated in 1 to 20, and strain 37 in 1 to 50. Normal horse serum is inactive above 1 to 20. Here again certain normal strains, namely 25, 45, and 138, were somewhat more sensitive and reacted in 1 to 50 to 1 to 100 dilutions.

Parameningococcus.—The polyvalent antimeningococcic horse serum was almost wholly inactive upon the two Dopter strains of parameningococci, while it agglutinated the two para-like strains 7 and 42 in dilutions 1 to 200. It should be recalled that the two latter strains were employed in the preparation of the serum.

The monovalent parameningococcus rabbit sera exhibited varying titers according as they acted upon the homologous or heterologous strains. With the former the limit was 1 to 500, with the latter 1 to 200. No difference was noted between the sera prepared from strains L or M (tables II and III).

On the other hand, the two para-like strains, 7 and 42, gave slightly different reactions according to the source of the immune parameningococcus serum. With para serum L, strain 42 agglutinated at 1 to 200, and strain 7 at 1 to 100; with para serum M, both agglutinated at 1 to 200. In other words, strains 7 and 42 behave as heterologous para strains against these two sera.

If we turn now to a monovalent serum prepared from normal strain 48 which agglutinated its own and two other normal strains in 1 to 500, the two Dopter para strains, L and M, agglutinated in 1 to 20, and the two para-like strains at 1 to 20 (strain 42) and 1 to 50 (strain 7) (table IV).

However, the Dopter para sera are not without agglutinating effects on normal strains of meningococcus, and both those that agglutinate regularly and irregularly. With para serum L, among the former, strain 18 reacts in 1 to 100, strains 25 and W in 1 to 50 dilutions; among the latter, strains 37 and BH react in 1 to 100,

strain 20 in 1 to 100 dilutions. With para serum M regular strains 18 and 49 react in dilutions of 1 to 100 and 1 to 50, respectively, irregular strains HP, I, and BH react in 1 to 200, and strains 9 and 20 in 1 to 100 dilutions.

TABLE IV.

Monovalent Rabbit Serum Immune to Meningococcus 48. Agglutination Reactions Made at 55° C.

Strains.	C	10	20	50	100	200	500
48	–	++	++	++	++	+	+
Para M	–	++	+	±	–	–	–
Para L	–	++	+	–	–	–	–
45	–	+	+	+	+	–	–
42	–	+	+	–	–	–	–
49	–	++	++	++	+	–	–
35	–	++	++	++	++	+	+
37	±	++	++	++	++	+	–
138	–	++	++	++	++	+	–
28	–	++	++	+	–	–	–
25	–	++	++	++	++	++	+
20	–	++	+	+	–	–	–
18	–	++	++	++	++	+	±
9	–	++	++	+	±	–	–
7	–	++	++	±	–	–	–
B	–	+	+	±	–	–	–
MA	–	+	+	+	+	–	–
HP	–	+	+	+	+	+	–
W	–	++	++	++	+	±	–
I	–	++	+	+	±	–	–
BH	–	++	+	+	+	+	–
F	–	++	++	++	+	–	–

The conclusion to be drawn from this series of tests is not to the effect that parameningococcus strains are strictly different as regards agglutination from normal strains of meningococcus, but that they nevertheless display a certain relative specificity.

Normal Meningococcus.—Two classes of normal meningococci have been recognized. They have been denominated "regular" and "irregular" according as they agglutinate in all or only in part of the normal immune sera. The variations in regard to agglutinability among normal strains are wide, as is exhibited in table V. It is this great variability that makes it impracticable on the basis of agglutination alone to separate certain strains as being a distinct group or species. It remains true, however, that the group distinguished by the name of "para" departs even more widely from the normal

standard than do the several irregular strains studied. And this difference reappears in respect to other immune reactions to be described.

TABLE V.

Agglutination Reaction at 55° C. Limit Dilutions for Complete Agglutination.

Strain.	Sera.				
	Board of Health.	Para M.	Para L.	Normal 48.	Normal 35.
Para L	1 : 10	1 : 100	1 : 500	1 : 20	1 : 10
Para M	1 : 10	1 : 500	1 : 200	1 : 20	1 : 20
Para-like 7	1 : 200	1 : 200	1 : 100	1 : 20	1 : 50
Para-like 42	1 : 100	1 : 200	1 : 200	1 : 20	1 : 50
Normal regular W	1 : 500	1 : 20	1 : 50	1 : 100	1 : 100
Normal regular F	1 : 50	1 : 20	1 : 20	1 : 100	1 : 50
Normal regular 18	1 : 50	1 : 100	1 : 100	1 : 200	1 : 100
Normal regular 25	1 : 50	1 : 20	1 : 50	1 : 500	1 : 200
Normal regular 28	1 : 100	1 : 20	1 : 10	1 : 50	1 : 50
Normal regular 35	1 : 10	1 : 10	1 : 10	1 : 500	1 : 500
Normal regular 45	1 : 50	1 : 50	1 : 10	1 : 100	1 : 200
Normal regular 48	1 : 50	1 : 10	1 : 20	1 : 500	1 : 50
Normal regular 49	1 : 100	1 : 50	1 : 20	1 : 100	1 : 100
Normal irregular BH	1 : 500	1 : 200	1 : 100	1 : 200	1 : 200
Normal irregular I	1 : 100	1 : 200	1 : 50	1 : 50	1 : 100
Normal irregular 9	1 : 200	1 : 100	1 : 50	1 : 50	1 : 50
Normal irregular 20	1 : 50	1 : 100	1 : 100	1 : 50	1 : 100
Normal irregular 37	1 : 100	1 : 50	1 : 100	1 : 200	1 : 100
Normal irregular 138	1 : 200	1 : 50	1 : 20	1 : 200	1 : 50
Normal irregular HP	1 : 20	1 : 200	1 : 50	1 : 200	1 : 200
Normal irregular MA	1 : 200	1 : 100	1 : 50	1 : 100	1 : 100
Inagglutinable B	1 : 20	1 : 10	0	1 : 20	0

Hence it is apparent that a clean cut classification into parameningococcus and meningococcus strains has been possible with thirteen only of the twenty-two strains whose agglutination reactions have been studied. The remaining nine act either irregularly or so nearly alike in all the sera tested that definite discrimination is not possible.

Attention is directed also to the fact that lack of agglutination by polyvalent antimeningococcic horse serum is insufficient evidence for the classification of meningococci into para and normal strains, since even normal meningococci do not invariably agglutinate in such a serum in high dilution, and some strains fail to agglutinate in dilutions greater than 1 to 20.

OPSONINS AND COMPLEMENT DEVIATION.

Opsonins.—The opsonin content is employed extensively for determining the therapeutic value of antimeningococcic serum. It was desirable therefore to test the specificity of this reaction upon normal and para strains of meningococci. For this purpose the Neufeld technique was employed. The result is shown in table VI, which tends again to isolate the two parameningococcus strains of Dopter from the strains of normal meningococcus employed.

TABLE VI.

Opsonization.

Serum.	Strain.	Control.	1:10	1:20	1:50	1:100	1:200	1:500	1:1,000	1:2,000	1:5,000
Board of Health	Para L	–	+	–	–	–	–	–	–	–	–
	Para M	–	+	–	–	–	–	–	–	–	–
	48	–	+	++	+	+	+	±	–	–	–
	35	±	++	+	+	±	–	–	–	–	–
	I	–	++	++	++	++	+	+	+	+	±
Fulminating case	48	–	+	+	+	+	+	±	–	–	–
	35	–	++	++	++	+	+	±	–	–	–
	Para L	–	+	+	+	–	–	–	–	–	–
	Para M	–	+	+	–	–	–	–	–	–	–
	I	–	+	+	+	±	–	–	–	–	–
Para M	Para M	–	+	+	+	+	+	+	–	–	–
	Para L	–	+	+	+	+	–	–	–	–	–
	48	–	+	+	±	–	–	–	–	–	–
	I	–	+	+	±	–	–	–	–	–	–
Para L	Para L	–	+	+	+	+	+	±	–	–	–
	Para M	–	+	+	+	+	–	–	–	–	–
	48	–	+	+	+	–	–	–	–	–	–
	I	–	+	±	–	–	–	–	–	–	–
Mild case	35	–	++	++	++	++	+	±	–	–	–
	48	–	++	++	+	+	±	–	–	–	–
	Para L	–	+	+	–	–	–	–	–	–	–
	Para M	–	+	+	–	–	–	–	–	–	–

Complement Deviation.—The degree of deviation of complement exerted by an antimeningococcic serum was recommended by Kolle and Wassermann to estimate its therapeutic value. Since, however, it appears that the reaction is subject to considerable and unexplained fluctuations it has not been generally adopted. None the less, it was desirable to determine the degree of specificity of the reaction as applied to para and normal strains.

Antigens were prepared by the method of Schwartz and McNeil.[9] When the antigens made with the parameningococci of Dopter were tested against the polyvalent antimeningococcic horse serum, complement was bound. When, however, the monovalent parameningococcic sera were titrated against normal meningococci, complement deviation occurred only in low dilutions. When the parameningococci antigens were titrated against monovalent sera prepared with normal meningococci strains 48 and 35, no binding was obtained. On the other hand, antigens of strains 48 and 35 deviated complement of the homologous sera in low dilutions (table VII). The action of the polyvalent antimeningococci serum corresponds with the results obtained by Dopter,[10] who, however, also noted that the sera of patients suffering from parameningococcal infection deviated complement in the presence of parameningococci but not of normal meningococci. If this is strictly true, then it must be held that the monovalent rabbit sera are less specific than the serum of patients.

The conclusion to be drawn from the tests of complement deviation is again to the effect that the para strains deviate from the normal strains, although the distinction cannot be said to be absolute.

PROTECTION EXPERIMENTS.

In the end the existence of profound differences between the para and normal strains of meningococci will be determined not so much by the immunity reactions already described, as by the results of protection tests, since in practice it is this test that determines whether special account should be taken of the para strains in the preparation of the antimeningococcic serum. For the purpose of the protection tests guinea pigs and monkeys were employed.

That guinea pigs weighing about 125 grams are especially susceptible to inoculation with cultures of the meningococcus was noted by Flexner.[11] I am able to confirm his observation. The tests were conducted with lethal doses by intraperitoneal injection of the

[9] Schwartz, H. J., and McNeil, A., *Am. Jour. Med. Sc.*, 1912, cxliv, 815.
[10] Dopter, *Bull. et mém. Soc. méd. d. hôp. de Paris*, 1911, xxxi, series 3, 590.
[11] Flexner, S., *Jour. Exper. Med.*, 1907, ix, 105.

TABLE VII.

Complement Deviation.

Complement, guinea pig serum, 1:40 dilution, in c.c.	Immune rabbit serum Para M, in c.c.	Para M antigen, in c.c.	Sheep corpuscles, 1:20 dilution, in c.c.	Anti-sheep rabbit serum, 1:100 dilution, in c.c.	Result.
0.1	0.1	0.25	0.1	0.1	No hemolysis.
0.1	0.2	0.25	0.1	0.1	No hemolysis.
0.1	0.1	0.15	0.1	0.1	No hemolysis.
		Culture 48 antigen.			
0.1	0.1	0.25	0.1	0.1	Incomplete hemolysis.
0.1	0.2	0.25	0.1	0.1	No hemolysis.
0.1	0.1	0.15	0.1	0.1	Complete hemolysis.
	Immune serum, fulminating case.				
0.1	0.1	0.25	0.1	0.1	No hemolysis.
0.1	0.1	0.15	0.1	0.1	No hemolysis.
0.1	0.01	0.25	0.1	0.1	No hemolysis.
		Culture 35 antigen.			
0.1	0.1	0.25	0.1	0.1	No hemolysis.
0.1	0.1	0.15	0.1	0.1	No hemolysis.
0.1	0.01	0.25	0.1	0.1	No hemolysis.
		Para L antigen.			
0.1	0.1	0.25	0.1	0.1	Complete hemolysis.
0.1	0.1	0.15	0.1	0.1	Complete hemolysis.
0.1	0.01	0.25	0.1	0.1	Complete hemolysis.
		Para M antigen.			
0.1	0.1	0.25	0.1	0.1	Complete hemolysis.
0.1	0.1	0.15	0.1	0.1	Complete hemolysis.
0.1	0.01	0.25	0.1	0.1	Complete hemolysis.
	Board of Health serum.				
0.1	0.1	0.25	0.1	0.1	No hemolysis.
0.1	0.1	0.15	0.1	0.1	Complete hemolysis.
0.1	0.01	0.25	0.1	0.1	Complete hemolysis.
		Para L antigen.			
0.1	0.1	0.25	0.1	0.1	No hemolysis.
0.1	0.1	0.15	0.1	0.1	No hemolysis.
0.1	0.01	0.25	0.1	0.1	Complete hemolysis.
		Culture 48 antigen.			
0.1	0.1	0.25	0.1	0.1	No hemolysis.
0.1	0.1	0.15	0.1	0.1	Incomplete hemolysis.
0.1	0.01	0.25	0.1	0.1	Incomplete hemolysis.
		Culture 35 antigen.			
0.1	0.1	0.25	0.1	0.1	No hemolysis.
0.1	0.1	0.15	0.1	0.1	No hemolysis.
0.1	0.01	0.25	0.1	0.1	Complete hemolysis.

several cultures alone and combined with homologous and heterologous sera. Preliminary experiments were made to determine the effects of normal rabbit and horse serum. They were found not to protect in corresponding doses against the cultures used. The cultures alone in the doses given invariably caused death. The following is a detailed example of a protective experiment in young guinea pigs, of which table VIII presents the results in brief.

Experiment 1.—A. May 27, 1914. Two guinea pigs weighing 120 gm. each were injected intraperitoneally with 0.2 c.c. of a 24-hour old sheep serum agar culture of Para M.

May 28. Both guinea pigs were dead.

B. May 27. Four guinea pigs weighing 120 gm. each were injected intraperitoneally with 0.2 c.c. of a 24-hour old sheep serum agar culture of Para M, followed at once by 0.5 c.c. of Para M immune rabbit serum.

May 28. Four guinea pigs were living but one was ill.

May 29. One guinea pig was dead.

C. May 27. Four guinea pigs weighing 120 gm. each were injected intraperitoneally with 0.2 c.c. of a 24-hour old sheep serum agar culture of Para M, followed at once by 0.5 c.c. of Para L immune rabbit serum.

May 28. All four guinea pigs were dead.

D. May 27. Four guinea pigs weighing 120 gm. each were injected intraperitoneally with 0.2 c.c. of a 24-hour old sheep serum agar culture of Para M, followed at once by 0.5 c.c. of immune rabbit serum 48.

May 29. Three guinea pigs were dead.

E. May 27. Four guinea pigs weighing 120 gm. each were injected intraperitoneally with 0.2 c.c. of a 24-hour old sheep serum agar culture of Para M, followed at once by 0.5 c.c. of immune rabbit serum 35.

May 28. All four guinea pigs were dead.

F. May 27. Four guinea pigs weighing 120 gm. each were injected intraperitoneally with 0.2 c.c. of a 24-hour old sheep serum agar culture of Para M, followed at once by 0.5 c.c. of Board of Health antimeningitis serum.

May 28. One guinea pig was dead.

What is noticeable is the general fluctuation of protection in that each monovalent serum, while being most perfectly protective for its homologous organism, exerts, also, some, if variable, amounts of protection against other or heterologous organisms. In this regard it cannot be said that the para strains of Dopter acted more regularly and specifically than the normal strains. On the whole, and as was probably to be expected, the Board of Health polyvalent serum showed the greatest regularity of action. In other words, this polyvalent serum carried protective immune bodies in about equal amount for the normal and para strains.

It was deemed desirable to ascertain the protective value of mono-

TABLE VIII.

Serum Protection Experiments.

Serum.	Cultures employed.				
	Para L.	Para M.	Culture 48.	Culture 35.	Culture 45.
Para L	Protected all	Protected none	Protected 8 of 10	Protected all	Protected none.
Para M	Protected 4 of 10	Protected 7 of 10	Protected 5 of 10	Protected none	Protected none.
Culture 48	Protected 6 of 10	Protected 4 of 10	Protected all	Protected 9 of 10	Protected none.
Culture 35	Protected 4 of 8	Protected none	Protected 5 of 6	Protected all	Protected none.
Board of Health	Protected 5 of 6	Protected 5 of 6	Protected 5 of 6	Protected 3 of 6	
Normal horse	Protected none	Protected none	Protected none	Protected none	
Normal rabbit	Protected none	Protected none	Protected none	Protected none	

valent sera upon monkeys infected by intraspinal inoculation and treated in the same manner. For this purpose parameningococcus L (Dopter) was employed for infection. The culture proved to be of low virulence, necessitating large doses in order to set up fatal infection. The method was to inject the culture and then immediately afterwards the immune sera. The following small series of experiments was made.

Experiment 1.—Control. A *Macacus rhesus* received intraspinally the surface growths of two sheep serum water agar slant cultures suspended in normal saline. Three hours after the injection the animal became ill; death occurred in twenty hours. At autopsy the meninges were congested and edematous; cultures of parameningococcus L were recovered.

Experiment 2.—A second *Macacus rhesus,* having received a similar dose of the suspended culture, was given five minutes later 1.5 c.c. of parameningococcus rabbit serum L. Slight symptoms of illness only developed. Twenty-four hours later lumbar puncture yielded turbid fluid containing polynuclear leucocytes enclosing diplococci; no diplococci were found outside of cells. A second dose of 1.5 c.c. of the immune serum was administered. At the expiration of another twenty-four hours the animal appeared well and the cerebrospinal fluid was clear.

Experiment 3.—A third *Macacus rhesus* was inoculated with the established dose of parameningococcus L and five minutes later was given 1.5 c.c. of immune rabbit serum prepared from normal meningococcus 48. No protection was afforded, and death occurred within twenty hours.

Experiment 4.—The fourth and last test was made with parameningococcus L and immune rabbit serum prepared with parameningococcus M. It was a repetition of experiment 2. The animal recovered completely.

The series of tests with monkeys is of value in supporting the group distinction between the normal and para meningococci. It is highly improbable that in a larger series of experiments some degree of cross-protection should not have been found between the normal and para organisms. On the other hand, the experiments indicate that para sera L and M are equally effective for protection against a given parameningococcus as in the case of para organism L.

DISCUSSION.

The study of *Diplococcus intracellularis* or meningococcus and allied organisms has led to the setting up of four classes as follows: (1) pseudomeningococci found by von Lingelsheim;[12] (2) diplococci derived from cases of posterior basilar meningitis described by Houston[13] and other English workers; (3) S strains isolated by Friese and Müller[14] from the nasopharynx of patients not having meningitis, and classified by Sachs-Müke[15] as pseudomeningococci; and finally (4) the diplococci described by Dopter as parameningococci. Von Lingelsheim's cocci are so readily differentiated from true meningococci by their morphological and cultural characteristics that they require no further mention. The other three classes, however, are described as being morphologically and culturally indistinguishable from true meningococcus, differing only in serum reactions, especial stress being laid upon differences in agglutinating power.

The diplococci from cases of basilar meningitis have been shown to be true meningococci,[16] a fact further substantiated by the tests

12 von Lingelsheim, W., *Klin. Jahrb.*, 1906, xv, 373.

13 Houston, T., and Rankin, J. C., *Brit. Med. Jour.*, 1907, ii, 1414.

14 Friese, H., and Müller, H., *Klin. Jahrb.*, 1909, xx, 321.

15 Sachs-Müke, *Klin. Jahrb.*, 1911, xxiv, 425.

16 Wollstein, M., *Jour. Exper. Med.*, 1909, xi, 579.

made in the present study with diplococci from two personal cases of chronic basilar meningitis, and by one strain sent by Dr. Houston in 1908.

The S cocci described by Friese and Müller were not obtained from meningitis patients nor from persons who had been in contact with cases of meningitis, and all the cultures differed markedly in agglutination reactions from strains of true meningococcus. In the absence of other serum tests it is not possible to bring these cocci into relation with parameningococci.

Dopter's parameningococci remain, then, in a class by themselves, differing serologically more or less from other diplococci. Although Dopter first found them in the nasal mucus of persons who had been in contact with meningitis patients, other observers soon demonstrated their presence in the blood and cerebrospinal fluid. Thus in 1910 a case of purpura fulminans with septicemia was observed by Carnot and Marie,[17] from which the organism was isolated from the blood. No meningitis was present in this case. Menetrier[18] was the first to report a case of meningitis due to parameningococcus. The patient was an infant and Menetrier noted a marked difference between the apparent mildness of the symptoms and the intensity of the infection as evidenced by the character of the cerebrospinal fluid, in which the majority of the cocci were extracellular. Injections of the usual antimeningococcic serum instead of causing amelioration of symptoms and fall in temperature were followed by intensification of symptoms. Seven cases of meningitis due to the parameningococcus having come under Dopter's observation, he recommended the therapeutic use of ordinary antimeningococcic serum to be followed later by an antiparameningococcic serum if laboratory examination showed the presence of parameningococcus infection. Dopter[19] prepared such an antiparameningococcus serum in 1912, and its use was followed by the recovery of cases of meningitis caused by the parameningococcus

[17] Carnot and Marie, P.-L., *Bull. et mém. Soc. méd. d. hôp. de Paris,* 1911, xxxi, series 3, 74.

[18] Menetrier, *Bull. et mém. Soc. méd. d. hôp. de Paris,* 1911, xxxi, series 3, 82.

[19] Dopter, *Semaine méd.,* 1912, xxxii, 298.

(Salin and Reilly,[20] Mery, Salin, and Wilborts,[21] Menetrier and Avezou,[22] and Hallé[23]). In these cases two or three injections of ordinary antimeningococci serum were said to have been without effect, while the injection of the antiparameningococcus serum was followed by prompt improvement. Hallé, noting that only extracellular cocci were present in the cerebrospinal fluid after the ordinary serum had been given, did not wait for cultures of the diplococcus before resorting to the antiparameningococcus serum. He believes that a mixture of para and true meningococcus serum will give good results in the treatment of meningitis, though he agrees with Netter[24] that polyvalent serum is best. Netter uses a mixture of the two sera, but believes that a polyvalent serum, like that made in America, and which has given him excellent results, fulfills all requirements.

SUMMARY AND CONCLUSIONS.

The parameningococci of Dopter are culturally indistinguishable from true or normal meningococci, but serologically they exhibit differences as regards agglutination, opsonization, and complement deviation.

Because of the variations and irregularities of serum reactions existing among otherwise normal strains of meningococci it does not seem either possible or desirable to separate the parameningococci into a strictly definite class. It appears desirable to consider them as constituting a special strain among meningococci not, however, wholly consistent in itself.

The distinctions in serum reactions between normal and para meningococci are supported by the differences in protective effects of the monovalent immune sera upon infection in guinea pigs and monkeys.

[20] Salin, H., and Reilly, J., *Bull. et mém. Soc. méd. d. hôp. de Paris,* 1913, xxxv, series 3, 423.

[21] Mery, H., Salin, H., and Wilborts, A., *Bull. et mém. Soc. méd. d hôp. de Paris,* 1913, xxxv, series 3, 411.

[22] Menetrier, P., and Avezou, J., *Bull. et mém. Soc. méd. d. hôp. de Paris,* 1914, xxxvii, series 3, 45.

[23] Hallé, *Bull. et mém. Soc. méd. d. hôp. de Paris,* 1914, xxxvii, series 3, 149.

[24] Netter, *Bull. et mém. Soc. méd. d. hôp. de Paris,* 1914, xxxvii, series 3, 53.

It is therefore concluded that it is highly desirable to employ strains of parameningococcus in the preparation of the usual polyvalent antimeningococcic serum. It remains to be determined whether it is better to employ the parameningococci along with normal meningococci in immunizing horses, or to employ normal and para strains separately in the immunization process and to combine afterwards, in certain proportions, the sera from the two kinds of immunized horses.

A STUDY OF THE METABOLISM OF CALCIUM, MAGNESIUM, SULPHUR, PHOSPHORUS, AND NITROGEN IN ACROMEGALY.*

By OLAF BERGEIM, Ph.D., F. T. STEWART, M.D., and P. B. HAWK, Ph.D.

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Marie (1) was the first to call attention to the relation of acromegaly to hypophyseal tumor, and he believed the disease to be a manifestation of the decreased function of this gland. This view was supported by various observers, but at the present time it has been largely given up and replaced by the theory of a hypersecretion of the anterior lobe as a necessary antecedent of acromegaly. Among the facts supporting this may be mentioned the following. All tumors of the hypophysis do not result in acromegaly, but only the adenomas. Destructive tumors do not evoke symptoms of the disease (Fischer (2) and others). This is supported by surgical experience. Hochenegg (3) first showed that removal of the hypophyseal tumor in a typical case of the disease could lead to a rapid recovery.

The apparently beneficial effects of pituitary extract administration have been due to alleviation of subjective symptoms and not to alterations in the fundamental growth disturbance (Biedl (4)). The conflict of views has been considerably clarified by the suggestion of Tamburini (5) that two pathological processes were involved, one leading to an hypertrophy of the gland and to acromegalic symptoms, and being followed by atrophic changes with cachectic symptoms. The widely varying results and differing conclusions as to the metabolism in acromegaly and the effects in general of the hypophysis upon nutrition are probably due to the failure to recognize the occurrence of different stages in the disease.

A retention of calcium and phosphorus has been found in acromegaly by Rubinraut (6), Edsall and Miller (7) in the progressive stage, Parhon (8), Medigreceanu and Kristeller (9), and Moraczewski (10). Tauszk and Vas (11) found an increased excretion of calcium but a slight retention of nitrogen and phosphorus. Franchini (12) obtained a retention of nitrogen, calcium, and magnesium, with a loss of phosphorus and chlorine. Schiff (13) found a retention of phosphorus in a case of acromegaly with myxedema. Oberndorfer (14) believes that there are no marked changes in metabolism. In his case there was a slight loss of calcium and a gain of phosphorus. Most of these authors found slight retentions of nitrogen and of chlorine. Medigreceanu and Kristeller noted a slight retention of sulphur, but Rubinraut found a loss

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of this element. It is apparent that the agreement of results is not so marked as to make further work unnecessary. If it be true, as seems probable, that there is a primary progressive stage of acromegaly and that this terminates in a resting or retrograde stage, some of these variations may be accounted for.

Work has been done to determine the effects of hypophysis treatment in this disease. Schiff found an increase in phosphorus excretion in acromegaly after treatment with pituitary tablets; in normal persons this was not noted. In a case complicated with glycosuria Moraczewski noted after similar treatment a negative nitrogen and chlorine balance with little change in calcium and phosphorus excretion. Rubinraut found that hypophysis feeding increased the excretion of calcium and phosphorus. Tauszk and Vas noted no particular effect. Medigreceanu and Kristeller in two successive periods of feeding of the anterior lobe noted distinct losses of phosphorus and of calcium, and a loss of nitrogen.

Similar experiments have been made on lower animals. Thompson and Johnston (15) fed dried glands to dogs and noted an increased excretion of nitrogen and phosphorus in the urine. Falta, Bolaffio, and Tedesko (16) found pituitrin to have the same effect as thyroid extract. It increased the elimination of calcium and magnesium through the feces. Oswald (17) observed no change in the nitrogen and phosphorus excretion of dogs. Malcolm (18), also working on dogs, found that feeding the posterior lobe caused a phosphorus loss, while the anterior lobe caused a nitrogen and phosphorus gain with a loss of calcium and magnesium. The administration of fresh gland had apparently nearly the opposite effect.

Working with rabbits Franchini (19) noted an increased excretion of calcium, magnesium, and phosphorus. Mochi (20) likewise by subcutaneous injections obtained increased elimination of calcium and phosphorus, which he ascribed to catabolism of bone.

In hypophysectomized animals there seems to be a greatly increased excretion of nitrogen and of phosphorus (Narboute (21) and Wolf and Sachs (22)).

Closely related to these findings are the effects on growth noted during hypophysis treatment. Cerletti (23) found that injection of extracts caused the body-weight to remain almost stationary. Sandri (24) also found a slowing in growth of mice fed on hypophyseal substance. Aldrich (25) noted no marked changes in young dogs. Schäfer (26) found that dried extract of the anterior lobe caused young rats to grow more rapidly.

The diversity of results obtained with gland extracts is not difficult to understand when we realize the different conditions under which they were obtained. The extracts were made in different ways and in most instances from mixtures of posterior and anterior lobes which possess distinct influences on metabolism. The results obtained with anterior lobe extracts should be of more value as this is the portion which normally influences growth, while the posterior portion may seriously disturb metabolism. The results in acromeg-

aly must also vary with the stages of the disease and other conditions.

The bone changes in acromegaly, according to Biedl, are not so marked as clinical appearances would suggest. There is no special disease process involved, but a deposition and resorption of bone substance takes place just as in normal bone growth.

The present study was made in the wards of the Jefferson Hospital on a man who was suffering from a typical early and progressive form of acromegaly.

The points of interest in the case reported may be briefly summarized as follows:

J. S. Male, aged 35 years, native of Russia; barber. Admitted to the Jefferson Hospital, Nov. 17, 1913, and discharged without change in condition, Dec. 9, 1913. He had never had any serious illness but had suffered during the past fifteen years from a catarrhal condition of the nose.

Physical Examination.—Well developed, well nourished, with typical acromegalic aspect. Head large and well developed. Eyes showed contracted pupils and reacted slowly to light and to accommodation. Nose large and broad at tip. Mouth and ears normal. No separation of upper teeth but slight separation of lower teeth especially about right lower canines. The arteries were hard and tortuous. The hands were very large, the fingers large and thick, and the ends blunt and sausage-like. Skin very thick. Sensation impaired in fingers, especially on dorsal surfaces.

Röntgen ray showed true acromegaly. The sella turcica was ⅞ of an inch long, and ⅞ of an inch deep. The posterior clinoid processes were somewhat atrophied.

Eye examination showed vision $o'' = 20/30$. The pupils reacted to light convergence. The tensions were normal, and ocular movements were unimpaired. The ophthalmoscopic examination was negative. Examination of nose and throat showed deflected septum and large submerged tonsils which were operative.

METHOD OF STUDY.

The details of food preparation and collection were carried out with the careful coöperation of the patient and of the hospital staff. The patient was placed on a uniform diet, amounts of each food given being based on the patient's choice during a preliminary day. The diet consisted of milk, graham and soda crackers, cereal, salt, sugar, water, butter, meat, tea, and coffee, and during the three final days also five grams of agar-agar per day. The meat was prepared previous to the experiment, thoroughly mixed, boiled, and

again thoroughly mixed, placed in glass jars, sterilized, and kept in a frozen condition until used. Aliquot portions of the foods were taken for analyses.

Urine was collected in exact twenty-four-hour samples and kept in an ice box. Thymol was used as a preservative. The feces were separated by the use of carmin and kept in thymolized friction top cans and in a frozen condition until the end of the period when they were thoroughly mixed for analysis.

Calcium and magnesium were determined by McCrudden's methods (27). Sulphur in the urine was determined by Benedict's method (28). Sulphur in the feces and foods was determined after oxidation with fuming nitric and hydrochloric acids and with Benedict's sulphur reagent. Phosphorus was determined by Neumann's method. Nitrogen was determined by the Kjeldahl method.

DISCUSSION.

The most striking fact brought out by the balance of the five elements in our case of acromegaly is the retention of calcium oxide, magnesium oxide, and phosphoric anhydride, of 16.2, 20.6, and 15.3 per cent. respectively (table I). The absolute retentions of

TABLE I.

Balance of Calcium, Magnesium, Phosphorus, Sulphur, and Nitrogen in Acromegaly.

	Calcium oxide.	Magnesium oxide.	Phosphoric anhydride.	Sulphur.	Nitrogen
	Grams.				
Ingestion daily	1.494	0.486	3.192	1.190	18.84
Excretion urine	0.159	0.160	1.701	1.006	17.60
Excretion feces	1.093	0.226	1.002	0.135	1.10
Excretion total	1.252	0.386	2.703	1.141	18.70
Retention daily	0.242	0.100	0.489	0.049	0.14
Retention per cent	16.2	20.6	15.3	4.1	0.7

these elements, 0.242 of a gram of calcium oxide, 0.100 of a gram of magnesium oxide, and 0.489 of a gram of phosphoric anhydride, cannot be considered high, but are nevertheless distinct. The fact that the sulphur and nitrogen metabolism shows a practical equilibrium emphasizes these retentions, as does also the fact that there is

a rough parallelism in the retentions of these bone-forming elements. There would seem, therefore, to be a disturbance in bone growth not secondary to changes in general metabolism, probably leading to increased formation of osseous substance.

Whether the bone thus formed be normal bone, as has been suggested, or abnormal bone, as believed by Edsall and Miller, cannot be decided from the data at hand. The fact that magnesium oxide and phosphorus were retained in amounts more than sufficient to form bone with the retained calcium oxide is of interest, as a similar condition was found by Edsall and Miller and others for the element phosphorus. Probably, however, too much emphasis cannot be laid upon these variations, and the most direct procedure here would be the analysis of bones from this condition.

TABLE II.

Daily Excretion of Calcium, Magnesium, Phosphorus, Sulphur, and Nitrogen in Acromegaly.

Urine.

	Day.							Daily average.
	1	2	3	4	5	6	7	
	Grams.							
Calcium oxide	0.064	0.131	0.163	0.190	0.085	0.225	0.256	0.159
Magnesium oxide	0.032	0.220	0.144	0.250	0 043	0.191	0.242	0.160
Phosphoric anhydride	1.672	1.972	1.572	1.666	1.577	1.715	1.735	1.701
Sulphur	1.004	1.009	1.003	1.010	1.003	1.008	1.003	1.006
Nitrogen	15.38	17.25	18.13	18.26	17.26	17.74	19.15	17.60

Feces.

Daily averages (grams).

Calcium oxide.	Magnesium oxide.	Phosphoric anhydride.	Sulphur.	Nitrogen.
1.093	0.226	1.002	0.135	1.10

The ratios of the distribution of the excretion of the mineral elements between the urine and the feces are for calcium oxide 1 to 6.9, for magnesium oxide 1 to 1.4, and for phosphoric anhydride 1.7 to 1. These are within normal limits and suggest that absorption and excretion of these elements are carried out in about the normal way. The same can probably be said for the elements sulphur and nitrogen, which are as usual eliminated almost entirely by way of the kidneys. That the absolute amounts of calcium oxide and mag-

nesium oxide eliminated by the urine are almost exactly identical is probably a coincidence.

A fact probably without particular relation to acromegaly but of great importance from the standpoint of general calcium and magnesium metabolism is brought out by table II. Pronounced variations in the daily urinary output of these elements will be noted, although the patient was on a strictly uniform diet, and the urine was collected at exact twenty-four-hour intervals. Further, the sulphur and nitrogen excretions are uniform. Nothing could show more clearly the dangers of judging the metabolism of these elements from urinary analyses alone. Take for example the excretion of calcium oxide on days 4, 5, and 6, 0.190, 0.085, and 0.225; and of magnesium oxide, 0.250, 0.043, and 0.191 on the same days. The explanation of these variations is not entirely clear, but the possibility has suggested itself as worthy of consideration that there is a relation between the absorption, particularly of calcium, and the accumulation in the intestine of residual material rich in salts soluble with difficulty. This would probably be particularly true on a diet containing considerable milk with its high content of calcium salts. In the study under discussion no feces were passed until the fifth day, and there is a steady rise in urinary calcium excretion until that day when there is a pronounced drop followed by marked rises on the succeeding days. It would seem desirable to consider this point in connection with studies of calcium metabolism, as, if supported further, it would tend to show reports of increased or decreased urinary output of these elements to be of very doubtful value. It may be, however, that these variations are particularly notable in acromegaly due to an increased absorptive power of the intestine for calcium salts in this condition.

The authors wish to express their obligation to Miss Gilson, Miss Looman, and Miss Troester for their aid during the progress of the investigation.

SUMMARY.

A distinct retention of calcium, magnesium, and phosphorus was noted, which was not accompanied by corresponding changes in general metabolism, as evidenced by a practical balance of nitrogen and sulphur.

It is concluded that there is a primary disturbance in the metabolism of the mineral elements mentioned, with the probable formation of new bony tissue.

The distribution of elements between urine and feces shows no abnormal variations.

A suggestion is made as to the reasons for variable calcium output in the urine on a uniform diet.

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CALCIUM METABOLISM AFTER THYRO-PARATHYROIDECTOMY.*

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That the thyroid apparatus, particularly the parathyroids, either directly or indirectly exerts considerable influence over the metabolism of calcium has been clearly shown in a number of ways by several authors. Probably the most striking of these experiments are those made by Erdheim (1) on rats. He observed that after parathyroidectomy the dentine of the rapidly growing incisor teeth ceased to calcify. After successful transplantation of the glands the calcification process again commenced and a zone of uncalcified dentine marked the period during which the parathyroids were absent. Erdheim and Canal (2, 3) also found that callus formation was greatly retarded, due almost entirely to deficient deposition of calcium salts. He also cites the relative frequency of parathyroid hyperplasia in osteomalacia, as well as changes in the teeth in this disease, similar to those noted by him in the animals mentioned.

Biedl (4) found that thyroidectomy prevented the normal development of the bony skeleton in animals, and Bircher (5) showed that treatment with thyroidin in these cases led to a more rapid formation of bone and a hastened process of calcification. Towles (6) found no special peculiarities in the calcium metabolism of exophthalmic goitre. Analyses of the blood and of various tissues have been made after parathyroid removal but these show a lack of uniformity. MacCallum and Voegtlin found a decrease of calcium in the blood and brain of dogs killed in parathyroid tetany, but not after incomplete removal (7). This has been confirmed by Pexa (8) and Aschenheim (9). Analyses by Cooke (10), however, indicate an increased calcium content of the brain under these conditions. This author found little change in the calcium metabolism. Leopold and von Reuss (11), in their studies of the calcium content of the tissues of normal and parathyroidectomized rats, found little change with adults and some decrease in young animals. The soft tissues were poorer in calcium and the bones were richer in this element. Similarly Morel (12) found that parathyroid extracts favored the growth of bone in young, but not in old animals. Neurath (13) found an increase in the calcium precipitable by oxalate in the blood in animals after parathyroidectomy. Most authors claim that an increased excretion of calcium in the urine follows this operation. Among these are MacCallum and Voegtlin (14) and Frouin (15). Musser and Goodman (16), however, found a marked decrease in the urinary calcium, as did also von Reuss and Welde. Cooke likewise found no increased excretion of calcium in the urine.

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The relation of calcium metabolism and the parathyroids has been studied most extensively in connection with the tetany brought on by their removal. The finding by MacCallum and Voegtlin that injection of calcium salts caused a temporary disappearance of the symptoms has been confirmed by a number of investigators. Some doubt is expressed as to any actual prolongation of life by this treatment. Arthus and Schaffermann (17) apparently obtained somewhat more persistent results in rabbits with calcium chloride ingestion. That this power is not restricted to calcium salts has been shown by Berkeley and Beebe (18) and by Canestro (19), who obtained similar results with magnesium and strontium salts, by Frouin (20) with lanthanum and thorium salts, and by Joseph and Meltzer (21) for sodium chloride solution. Further, simple bleeding decreases the symptoms. On the other hand, it has been shown that the injection of calcium precipitants causes a more rapid oncome of tetany (22).

Numerous attempts have been made to correlate the above findings with the tetanies of man presumably of parathyroid origin. In infantile tetany von Cybulski (23) found a much less calcium retention during the period of tetany. Schwarz and Bass (24) obtained similar results. Schabad (25) in a case of rachitis with tetany found no changes from the metabolism of rachitis alone, nor were the symptoms alleviated by treatment with calcium salts. Phosphorized oil, however, caused a retention of calcium and an improvement of the tetany. Haskins and Gerstenberger (26) could not show that in man parathyroid and calcium salts favored calcium retention. The theory of Stoeltzner (27) that tetany is due to a heaping up of calcium in the organism has met with much objection. On the other hand, Cattaneo (28) found a decreased calcium content of the blood in six cases of spasmophilia, and it has been suggested that the tetanies of pregnancy may be due to the drain on calcium that occurs at this period. Parathyroid tetany also develops very quickly in lactating animals (Carlson (29)).

The therapeutic application of calcium treatment of tetany in human beings has met with some success (30, 31, 32). The relation of infantile tetany to calcium metabolism is further supported by the work of Quest (33) who found a low calcium content of the brains of children who died in tetany. This was confirmed by Silvestri (34) but not by Cohn (35) or by Leopold and von Reuss.

With regard to the inevitability of the oncome of tetanic symptoms in various animals and in man after complete removal of the thyroid apparatus there has been much dispute, although the opinion more generally held seems to be that the results are always fatal and that any other termination is to be ascribed to the incompleteness of the operation, or insufficient length of observation. Much evidence has, however, accumulated in support of the view, emphasized particularly by Vincent (36), that a considerable percentage of animals of different species may survive total extirpation for prolonged periods and may show no specific symptoms of any kind; and also that the operation produces very different results in different species, in general the carnivora being much more sensitive than herbivorous animals. Vincent concluded that in monkeys neither thyroids nor parathyroids were essential to life. Breisacher (37) found that dogs fed on a milk diet suffered less than those on a meat diet. Vassale and Generali (38) found further that the tetany induced by thyroidectomy is

less marked in old than in young animals. In hunger the tetany was much less marked also than on a meat diet.

Marine (39) apparently goes so far in the belief that the parathyroids are essential as to make the absence of symptoms after removal a test for the presence of accessory glands. Parathyroidectomy extending over a long period was less often fatal and calcium salts sometimes tided an animal over an otherwise fatal tetany. Why this should be taken as a sign of compensation by accessory tissue rather than by some other gland or set of glands does not seem clear. Paton (40) has suggested that "the variations in symptoms may be due to the fact that removal of parathyroid reduces the stability of the central nervous system but that some other disturbing factor, for example, pregnancy, is required to upset it sufficiently to cause the manifestation of the symptoms."

The observations of Gozzi (41) and others (42, 43), that thyroparathyroidectomy was less rapidly fatal than removal of parathyroids alone, do not agree with the findings of Halpenny (44), Wiener (45), and Biedl (46). Other work done along this line will be mentioned in the discussion of our results.

In connection with the calcium metabolism the findings of Greenwald (47) should be mentioned. This author found an increase in the total phosphorus of the blood and serum after parathyroidectomy, the phosphorus being mainly in an ether-insoluble, acid-soluble form. He found likewise (48) a decrease of the phosphorus of the urine after operation with an increase as tetany came on. A pronounced increase in the creatinin content of the urine was noted. Frontali later observed (49) a decreased urinary creatin output after thyroidectomy. The latter also found a decrease of creatinin in the muscles and an increase in the blood.

Considerable study has been made of the nitrogenous metabolism after parathyroidectomy, particularly with the idea in view of proving or disproving an intoxication as the basis of the symptoms.

MacCallum and Voegtlin found a great increase in the ammonia content of the blood. Similar results were obtained by Cooke, Morel (50), and Medwedew (51), but could not be confirmed by Carlson and Jacobson (52), Albertoni (53), Greenwald, Musser and Goodman, and von Reuss and Welde. Increases of the ammonia content of the urine have been found by Berkeley and Beebe, Frouin (54), Cooke, and Morel. Greenwald found only a slight increase of ammonia but a larger increase of nitrogen of unknown character. Other abnormalities noted by Cooke were increases in the excretion of magnesium, and the presence of lactic acid due probably to the muscular work of tetany.

After thyroidectomy alone there is usually a reduction of organic as well as inorganic metabolism. However, Falta, Bolaffio, and Tedesko (55) found a pronounced increase in the nitrogen: phosphoric anhydride ratio, due to increased nitrogen excretion and decreased phosphorus excretion in the urine. Thyroidin treatment brought about an increased excretion of calcium in the feces. Similarly Scholz (56) found in man that such treatment brought about an increased phosphorus excretion in the feces. Kottmann (57) found a lowering of the freezing point of the blood in Basedow's disease, due supposedly to abnormal content of metabolic products.

The present study was carried out on a patient in the Jefferson Hospital.

Clinical History.—G. H., aged 32 years, colored; laborer. Admitted to the surgical ward of the Jefferson Hospital, Oct. 19, 1913. Fourteen months before this he began to experience difficulty in breathing, which increased steadily for two months, when a low tracheotomy was performed by a surgeon in Florida, where the patient was then residing. The condition evidently was thought to be syphilis of the larynx, as the patient was subsequently given salvarsan, and later neosalvarsan, potassium iodide, and inunctions of mercury. After coming to Philadelphia the patient entered Dr. D. Braden Kyle's ward at the Jefferson Hospital. Dr. Kyle found a growth in the larynx and removed a portion of this growth for microscopic examination. Histologic study of the excised fragment revealed a typical picture of papilloma. From a clinical standpoint, however, Dr. Kyle regarded the growth as malignant, and because of its extent transferred the patient to the surgical ward. There was nothing relevant in the family history. The patient had had gonorrhea about five years before his laryngeal trouble, but had never had a chancre or other evidence of syphilis. The Wassermann reaction was negative. There had never been much pain, never any hematemesis. The tracheotomy tube was worn constantly, and when this was closed with a finger the patient was able to whisper. The man was well nourished; the temperature, pulse, and respiration were normal. The pupils reacted to light and accommodation. There was a large cauliflower-like mass filling the glottis and extending up into the epiglottis and into the esophagus. On palpation the larynx was found to be considerably expanded, measuring three inches at the widest part. It could be moved laterally, and it moved when the patient swallowed. The thyroid gland was enlarged and hardened. Examination of the thorax, abdomen, extremities, and urine showed nothing abnormal.

An operation was performed on Oct. 21, 1913, intratracheal insufflation of ether being employed to maintain anesthesia. A median incision was made from above the hyoid bone to the tracheotomy opening, and a transverse cut made at each end of this incision, thus forming lateral flaps, which were turned outward. After separating the larynx from the soft parts on each side, it was discovered that the growth had invaded the thyroid gland, the trachea, and the esophagus. The sternohyoid and sternothyroid muscles were severed near the sternum, the omohyoid beneath the sternomastoid, and the trachea in the episternal notch below the tracheotomy opening. A tube was then passed into the esophagus through the mouth, and the anterior half of the esophagus, from the sternum to the pharynx, separated by sharp dissection while the specimen was dragged upwards to facilitate the incisions in the esophagus, which incisions were continued up into the pharynx to meet in the median line above the hyoid bone. The excised mass of tissue included a portion of the pharynx, the anterior half of the esophagus as low as the sternum, the hyoid bone, the epiglottis, the larynx, the trachea as far as the sternum, the entire thyroid gland, and of necessity the parathyroid glands, although it must be stated that owing to the neoplastic infiltration neither Dr.

Coplin nor myself could identify the parathyroids in the excised tissue. After the growth had been removed, the esophagus and the pharynx were sutured over the tube with catgut, and the skin flaps brought together except at the points corresponding to the outer angles of the lower transverse incision, where gauze drains were inserted. The tracheal stump was anchored to the skin with heavy silk thread. The operation lasted one and one half hours, and was followed by little shock. The patient sat up the next day and was out of bed at the end of the first week. Liquid food was given through a tube which reached to a point just below the lowest suture in the esophagus. On the eighth day the lower part of the esophageal wound began to leak and some of the food passed into the trachea. Subsequent to this nourishment was administered through a long tube which passed through the nose, pharynx, and esophagus into the stomach. The tube was not removed between feedings, because of the difficulty of guiding it past the opening in the lower cervical esophagus. During the first postoperative week the patient coughed only occasionally, but subsequent to the entrance of food into the trachea the cough became more frequent, the expectoration being tough stringy mucus. On Nov. 4, 1913, two weeks after the operation, sharp pain due to pleuritis was felt in the right chest. The respirations increased in frequency and fever developed. Later signs of fluid in the pleural cavity became evident, but none could be obtained by aspiration. On Nov. 21, 1913, an incision was made into the pleural cavity and a large quantity of foul pus evacuated. One week after this and thirty-eight days after the laryngectomy the patient died.

Autopsy.—A septic pneumonia of the right lung and gangrene of the right pleura were revealed. The laryngeal growth proved on microscopic examination to be a squamous-celled epithelioma.

METHOD OF STUDY.

The patient was kept in a private room in the Jefferson Hospital and under charge of a private nurse throughout the period of study.

On account of the necessity of feeding the patient through a nasal tube the food given was all in liquid form. It consisted of soup, eggs, milk, orange juice, and water. No attempt was made to have the amount of each food uniform from day to day but the amounts fed were accurately measured and aliquot portions were taken at each meal for analysis. The food samples for the day were mixed in the proportions as fed, evaporated to dryness, ground to a fine powder in a mortar, and thoroughly mixed. Portions of these powders were taken for analysis. Water was analyzed separately. The urine was carefully collected in twenty-four-hour periods, and calcium determinations were made in each day's output. Feces separation was made by means of carmin and was not attended with

difficulty. Feces were kept in a friction top can in a frozen condition until the end of the period when they were thoroughly mixed and portions taken for analysis. McCrudden's (58) method for the determination of calcium was employed.

Blood samples of fifty cubic centimeters each were obtained on the first and final days of the test period. Coagulation was prevented by means of potassium oxalate. The calcium was determined gravimetrically with platinum crucibles, by a modification of McCrudden's method for pure solutions.

The results obtained are given in table I.

TABLE I.

Ten-Day Calcium Balance after Thyroparathyroidectomy.

Day.	Calcium oxide in food.	Calcium oxide in urine.		Calcium oxide in feces.	Total excretion.
		Volume.	Calcium oxide.		
1	1.0923 gm.	635 c.c.	0.0051 gm.		
2	0.7610 gm.	870 c c.	0.0074 gm.		
3	1.6355 gm.	865 c c.	0.0091 gm.		
4	1.5258 gm.	900 c.c.	0.0131 gm.		
5	0.7036 gm.	740 c c.	0.0078 gm.		
6	1.6912 gm.	1,160 c c.	0.0145 gm.		
7	2.3261 gm.	940 c.c.	0.0193 gm.		
8	2.2882 gm.	1,125 c.c.	0.0169 gm.		
9	1.9273 gm.	1,110 c.c.	0.0244 gm.		
10	2.7847 gm.	1,765 c.c.	0.0159 gm.		
Period	16.7357 gm.		0.1335 gm.	16.1444 gm.	16.2779 gm.
Daily average...	1.6736 gm.		0.0134 gm.	1.6144 gm.	1.6278 gm.

Balance.
10 days .... 0.4578 gm. of calcium oxide retained.
Balance.
Daily average ...... 0.0458 gm. of calcium oxide retained.
Retention .... 2.74 per cent. of calcium oxide.

Blood.

First sample... 0.0087 per cent. of calcium oxide.
Second sample. 0.0100 per cent. of calcium oxide.

DISCUSSION.

It will be noted that there was a retention during the ten-day period of 0.4578 of a gram of calcium oxide, or 0.0458 of a gram per day. This corresponds to a percentage retention of 2.74 per cent. of an ingestion of 1.6736 grams of calcium oxide per day. A retention as slight as this on a comparatively high intake cannot be said to show after operation a distinct tendency of the body to take up

calcium salts. In fact it is probable that a normal individual on such a diet would tend to retain more rather than less calcium oxide than was noted in this case. The figures must therefore be taken to show an approximate calcium equilibrium. They have a certain negative significance in that they certainly give no support to any assumption of a pronounced calcium loss to the body in this condition.

The relation of the excretion of calcium in the urine to that in the feces was found to be approximately 1 to 121. This proportion is lower than was found by us in osteitis deformans or by McCrudden and Fales (59) in intestinal infantilism. It is in fact one of the most striking facts brought out by this table. Taken in connection with the low figures for calcium in the blood the most plausible interpretation is that absorption of calcium by the intestine must have been very slight. That there was a demand by the body for calcium salts to fight off infection or toxemia is indicated by the fact that three times as much calcium was retained as was excreted in the urine, small as this amount was. There must have been deficient absorption which must be attributed to the thyroid and parathyroid removal. How such an effect could be produced is problematical. In the apparently somewhat related intestinal infantilism McCrudden and Fales could not find that the poor absorption was secondary to the overproduction of phosphate, fatty or volatile acids, nor could they determine the form in which about one half of the calcium of the feces was combined. Of course the diet employed in our case, consisting largely of eggs and milk, contained considerable organic phosphorus and the possibility of a faulty digestion of these substances on calcium absorption must be considered, particularly in view of Dibbelt's experiments (60). He found that the introduction of sodium bicarbonate or disodium phosphate directly into the intestine of animals caused an increased excretion of calcium in the urine. He assumed that excessive amounts of these calcium-precipitating agents might be formed by decomposition of undigested casein and from carbohydrate fermentation and believes that the etiology of rachitis must be of this nature. We are not, however, aware of any evidence proving that this actually occurs. The fact that Keeton (61) has shown in

cats that parathyroidectomy greatly diminishes gastric secretion is of interest in this connection. A lack of hydrochloric acid secretion might operate in two ways to decrease calcium absorption. First, it decreases the amount of calcium chloride or acid phosphate formed from the carbonate or phosphate of the diet. Secondly, intestinal fermentation and putrefaction processes of a nature calculated to form calcium precipitants would be increased due to the decreased antiseptic power of the gastric juice. It would be of interest to know whether an achylia exists in intestinal infantilism, rickets, and allied disorders. It is our intention to investigate this point.

It must be borne in mind, however, that we know little with regard to the mechanism of calcium absorption. If we assumed that calcium was absorbed largely as chloride or acid phosphate in the stomach or upper intestine, the problem would be simplified. We have, however, noted in a study on acromegaly (61) that urinary excretion of calcium increases when calcium-rich feces accumulate in the intestine, indicating absorption of this element from the large intestine. It appears that in the present state of our knowledge we cannot bar out a more direct parathyroid action, through the intermediary of a nuclein metabolism or on the intestines themselves.

The slight increase in urinary calcium during the period may be considered as largely due to a higher calcium ingestion, possibly also to a slight constipation. The somewhat higher blood calcium content at the close of the period may be attributed to similar causes. Possibly also there has been a greater demand on calcium for antitoxic purposes and deficient excretion of calcium phosphate.

Why in the case of complete thyroid and parathyroid removal under consideration did not tetanic symptoms develop during the period of thirty-nine days after operation? Some authors who have worked particularly upon carnivorous animals, such as the dog, would unhesitatingly answer that accessory parathyroids must have been present. From the nature of such an assertion, precluding, as it does, either proof or disproof, and assuming as it does the point in question, it follows that this argument must be given not first but last consideration, and resorted to only if other explana-

tions are not forthcoming. Unfortunately the explanation of the absence of tetany in the present case cannot be given with any certainty. Possible explanations only can be suggested.

Our patient was on a high calcium diet. Absorption was not satisfactory, but there was a retention and not a loss of this element. Certainly these facts are significant. Calcium salts delay the oncome of tetany. Most observers have, however, found this inhibiting effect of calcium salts to be temporary. It could hardly be expected to extend over a period of more than a month.

The patient was further kept quiet and thus untoward influences were avoided. The suggestion of Paton that parathyroid removal reduces the stability of the central nervous system, but that other disturbing factors may be required to upset it sufficiently to cause symptoms, has already been mentioned. The facts that carnivora are more sensitive than herbivora, that dogs fed on a milk diet suffered less than on a meat diet, and that in old animals the tetany was less marked than in young animals, have already been considered.

Bircher (62) has reported complete and permanent recoveries from tetany following partial removal of thyroids and parathyroids, by means of temporary treatment with parathyroid extracts. Schneider (63) reports a case of neck tumor of the thyroid in a woman of 46 years, in which apparently complete thyroid and parathyroid removal was followed by acute tetany which was overcome by treatment with parathyroid extracts. This case as well as ours may of course be explained on the basis of the supposed presence of supernumerary parathyroids. It would seem more plausible that this compensation was brought about by hypertrophy of some other of the ductless glands, probably the hypophysis. This view is supported by the known relation of this gland to calcium metabolism, its rapid hypertrophy in the calcium-mobilizing period of pregnancy, and after thyroid removal, as shown by Rogowitsch (64) and others (65); also after parathyroid removal as reported by Thompson (66), Pepere (67), and Halpenny (68). It may be more than an interesting coincidence that hypophysectomy (Crowe, Cushing, and Homans (69)) is much more rapidly fatal in old than in young animals, while parathyroidectomy (Vassale and Generali (70) and others) has a higher mortality in the young when apparently the compensatory mechanism is not so well developed. Ott and Scott (71) have further found that pituitary extracts temporarily inhibit parathyroid tetany. The observations of Thompson, Leighton, and Swarts (72) are of interest in this connection. They found that ligatures producing slow interference with the blood supply led to no tetanic symptoms, but that slow wasting occurred.

The fact that the parathyroids in our case were so infiltrated as

to be unrecognizable is very important as suggesting that there had probably been a gradual decrease in parathyroid activity with sufficient lapse of time for a compensatory mechanism to develop sufficient to prevent the oncome of tetany at least for a considerable period of time, but not sufficient to preserve a normal metabolism of calcium.

The authors wish to express their indebtedness to Dr. Kinnaird, Miss Looman, and Miss Rowe for their coöperation.

CONCLUSIONS.

The metabolism of calcium was studied in a man after complete removal of the thyroid and parathyroid glands. A slight retention of calcium (0.4578 of a gram of calcium oxide in the ten-day period) was noted. The urinary calcium excretion was low, averaging 0.0134 of a gram per day on a daily ingestion averaging 1.6736 grams of calcium oxide. A slight increase was observed during the period of study in the calcium content of the blood.

No symptoms of tetany were noted in the patient, who survived operation thirty-nine days.

The low urinary and blood calcium values are taken to show deficient absorption of calcium, which may bear some relation to the decreased gastric secretion after parathyroidectomy.

Attempts are made to explain the non-occurrence of tetany as due to the high calcium intake and to the development of a compensatory mechanism in which the pituitary body may play a part.

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A METHOD FOR ESTIMATING THE BACTERIA IN THE CIRCULATING BLOOD IN RABBITS.*

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Not a few of the determinations in experimental bacteriology have to do with the testing of bacteria for their infectious power. The laboratory animals principally employed for this purpose are small, and afford few reliable criteria which can be used to decide the result. The criterion which, because of its very definite character, is usually relied upon is the development of severe illness and the intervention of death; but this criterion cannot take account of degrees of intensity of infection, except as they affect the period of survival of the animal after inoculation, which is manifestly a coarse differential. All who have conducted so called virulence tests with bacteria have felt the need of other means of differentiation than mere survival or death of the inoculated animal. In the course of some experiments on the therapeutics of streptococcal and pneumococcal infections, this need became imperative, so that a means was sought that should yield data capable of comparison at different periods and intervals after inoculation, and which did not rely alone on the ultimate result of recovery or death.

The starting point of the quest was the well known phenomenon, first observed by von Fodor[1] and investigated accurately by Wyssokowitsch,[2] namely, that bacteria injected into the circulation are soon filtered out of the blood and, according to their degree of infectiousness, are destroyed or reappear, multiply, and, when of sufficient power, ultimately cause death. Hence the question which arose was whether the disappearance, reappearance, and multiplication proceed with a degree of regularity and consistency rendering a numerical estimate feasible of the bacteria in the circulating blood.

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[1] von Fodor, J., *Arch. f. Hyg.*, 1886, iv, 130.

[2] Wyssokowitsch, W., *Ztschr. f. Hyg. u. Infectionskrankh.*, 1886, i, 3.

The later studies of Weil[3] and of Reichstein,[4] which deal with the fate and the estimation of streptococci within the blood stream, offered an encouraging affirmative on this point, so that the next question to arise was whether blood taken repeatedly from superficial vessels sufficed for the purposes of the estimation.

EXPERIMENTAL PART.

Bacteria and Injections.—The streptococcus used in the following experiments was originally isolated from the throat of a scarlet fever patient. It is a typical hemolytic, Gram-positive, chain-forming streptococcus. It has been repeatedly passed through rabbits and, at the beginning of this work, 0.25 of a cubic centimeter of a twenty-four-hour bouillon culture[5] per kilo of body-weight killed rabbits within one to five days. The pneumococcus used belongs to group I according to Neufeld's classification. Its virulence has been maintained by repeated passages through mice and rabbits. 0.001 of a cubic centimeter of a twenty-four-hour bouillon culture per kilo of body-weight kills rabbits within one to three days. In all experiments the bacteria were injected into the ear veins.

Taking the Blood.—The uninjected ear was carefully shaved and washed with 95 per cent. alcohol. The marginal vein was pricked and a few drops of blood allowed to flow out before any was taken for the cultures. Then a definite number of drops were caught directly into sterile Petri dishes. A tube of agar, previously melted and cooled to 42° C., was immediately poured into the dishes and the blood and agar were thoroughly mixed. Two or more plates

[3] Weil, E., *Ztschr. f. Hyg. u. Infectionskrankh.*, 1911, lxviii, 346.

[4] Reichstein, S., *Centralbl. f. Bakteriol., 1te Abt., Orig.*, 1913, lxxiii, 209.

[5] We have found blood bouillon (3 to 5 drops of sterile defibrinated rabbit blood in a tube of 5 c.c. of beef infusion bouillon) to be an excellent medium for streptococci and pneumococci. The difficulty of "infecting" large quantities of medium with loop inoculations from bouillon to bouillon is eliminated (Gillespie). The bacteria do not lose this virulence as readily in this medium as in ordinary bouillon. Another advantage is that the bacteria live in this medium for long periods of time without being transferred. Streptococci will live for 6 months when the cultures are kept in the dark at low temperature; it is not necessary to seal the tubes in any way. Pneumococci will live for at least 6 weeks. This medium was used throughout the experiments instead of plain bouillon.

were made from each rabbit, the amount of blood varying from one to ten drops, according to the supposed degree of infection. In this way plates were obtained on which the colonies could be easily counted and the various plates compared.

The above procedure permits of frequent taking of blood with the least possible disturbance to the rabbit. Contaminations rarely occur if the ear is properly cleansed and a few drops are allowed to flow out before taking the blood for the cultures. Blood from the first few drops adheres to the surface of the ear and the following drops run over this without coming in contact with the skin. The accuracy of the method might be objected to, because the measuring of the blood is as inaccurate as the size of one drop of blood may differ from another. If the veins are pricked by a stab with the same sharp-pointed instrument and the surface of the ear is held in a perpendicular position so as not to allow the blood to collect in large quantities before flowing off, drops of fairly constant size can be obtained and the number of colonies on various plates of the same number of drops is remarkably uniform. The accuracy of the procedure can also be tested by infecting a series of rabbits with the same quantity of a bacterial suspension per kilo of body-weight and taking cultures at stated intervals in a short time after the injections. Experiment 1, as given below, was done with this object in view.

STREPTOCOCCUS INFECTIONS.

Experiment 1.—Each of seven rabbits was injected intravenously with 0.25 c.c. of a fresh bouillon culture of streptococci per kilo of body-weight. Cultures were made from the opposite ear 30 minutes and 5½ hours afterwards. In this, as in all other experiments, the number of colonies was calculated on the basis of ten drops of blood. The results are given in table I.

TABLE I.

	Rabbit 1.	Rabbit 2.	Rabbit 3.	Rabbit 4.	Rabbit 5.	Rabbit 6.	Rabbit 7.
Colonies at 30 min..........	140	145	135	100	130	130	100
Colonies at 5½ hrs.........	8	9	20	22	10	12	10

Many experiments of this character were performed and similar results were always obtained. The first cultures were made after

ample time had elapsed for a thorough and uniform distribution of the bacteria in the blood stream and the number of colonies obtained from the different rabbits shows only slight variations. At the next bleeding, five hours later, the plates were still uniform, although a great reduction in the number of colonies had occurred. These results are probably due to the fact that this initial disappearance of the bacteria from the blood depends largely upon mechanical forces which are about the same in each animal. A biological reaction between the host and the infecting organism had evidently played a very slight rôle up to this time; otherwise, such uniform results could not be expected. Such an experiment also shows that this method of taking blood gives dependable data.

In following the progress of the infections further, it was found that they may take one of three courses, mainly depending upon the virulence of the bacteria. A series of experiments was carried out just after the bacteria had been passed through a number of rabbits. The infections ran an acute course, the blood was never free of bacteria, and the autopsies showed no evidence of localization. After two months' cultivation on artificial media, no animal passages being made, the same amount of bacteria caused a chronic infection in a majority of the animals. There were longer or shorter intervals during which the septicemias were slight or entirely absent. After a time the bacteria suddenly reappeared in the blood and the animals died within a few hours to two days. Autopsies showed localized infections, from which the second blood invasion undoubtedly came. When the bacteria were still less virulent, they permanently disappeared from the blood and the animals recovered. Examples of these types of infection are reported in the following tables.

Experiment 2.—Each rabbit was given 0.25 c.c. per kilo of body-weight of the ame suspension of streptococci into the ear vein. The blood for the cultures was taken from the opposite ear at the times indicated in the protocols. The number of colonies was estimated on the basis of ten drops of blood.

An examination of table II shows that the infections, with the exception of rabbits 2 and 3, ran a remarkably uniform course. From the time of the injections to about five hours afterwards there is a rapid decrease in the number of bacteria. From five to six

TABLE II.[6]

Animal.	Time of bleeding and number of colonies.											
Rabbit 1,	30 min.	2 hrs.	6 hrs.	10 hrs.	22 hrs.	48 hrs.	72 hrs.	Died 1 hr. after the last bleeding.				
weight 1,800 gm.	52	1	18	12	9	100	×					
Rabbit 2,	30 min.	2 hrs.	6 hrs.	11 hrs.	23 hrs.	30 hrs.	47 hrs.	56 hrs.	Was dead at 64 hrs.			
weight 2,000 gm.	200	5	100	200	1000	×	×	950				
Rabbit 3,	30 min.	2 hrs.	6 hrs.	10 hrs.	22 hrs.	48 hrs.	72 hrs.	96 hrs.	120 hrs.	126 hrs.	138 hrs.	Dead at 144 hrs.
weight 1,600 gm.	110	9	13	35	65	1500	×	100	75	×	100	
Rabbit 4,	30 min.	6 hrs.	18 hrs.	Dead at 36 hrs.								
weight 1,500 gm.	25	100	2000									
Rabbit 5,	30 min.	2 hrs.	6 hrs.	11 hrs.	23 hrs.	30 hrs.	36 hrs.	Dead at 38 hrs.				
weight 1,750 gm.	75	5	70	245	×	×	×					
Rabbit 6,	30 min.	3 hrs.	18 hrs.	24 hrs.	42 hrs.	48 hrs.	Dead at 50 hrs.					
weight 1,100 gm.	50	6	1000	2000	×	×						

[6] In the tables, × indicates that the plates were so heavy that the colonies could not be counted.

hours afterwards the bacteria begin to increase in number and septicemia becomes heavier and heavier until the death of the animal. The rabbits died in from one and a half to three days. Rabbits 2 and 3, especially 3, showed some variations from this rapid, progressive course. Rabbit 3 lived six days and its blood cultures give a possible explanation for this. At seventy-two hours the blood had reached so great a septicemic state that the animal should have succumbed on the following day. But the rabbit lived and the blood culture showed a retrogression of the infection for two days. This was followed by another exacerbation which was also overcome and succeeded by another retrogression. The rabbit died on the sixth day with a low culture; but six hours elapsed between the last bleeding and the death of the animal. This gave sufficient time for another ascension, which probably occurred, and as a result of which the rabbit died. Such fluctuations or crises cannot be ascribed to the technique, since they were often observed, and also a temporary reduction in the number of bacteria was always accompanied by a prolongation of the rabbit's life. Infections running such zigzag courses are positive indications that a two-sided warfare occurs within the body of the host; the bacteria make advances which are successfully met by the defensive power of the rabbit, but, not being entirely killed out, they make another trial. Such a struggle may be prolonged for several days, and the final results depend upon the relative strengths of the aggressive forces of the invading bacteria and the defensive resources of the infected animal.

Experiment 3.—This experiment was carried out two months after experiment 1. The same strain of streptococcus was used in this experiment and the same quantities of bouillon culture were injected. This strain had been kept on artificial media continuously and had evidently suffered a loss of virulence. The technique was the same as in experiment 1. Two typical rabbits are reported in table III.

The courses of the infections reported in table III show that the bacteria had lost in aggressiveness but they still possessed an insusceptibility to the destructive influences of the host and were yet able to carry on a local fight in some more or less inaccessible place. The local lesions probably lowered the vitality of the animals to

TABLE III.

Animal.	Time of bleeding and number of colonies.
Rabbit 1,	30 min. 2 hrs. 6 hrs. 24 hrs. 48 hrs. 72 hrs. 96 hrs. 120 hrs. 140 hrs. 144 hrs. 168 hrs. 192 hrs.,
weight 1,540 gm.	76 0 0 0 0 0 0 0 0 0 150 rabbit died.
Autopsy.	Pleurisy with effusion. Streptococci abundant in the fluid. Smear from the heart's blood heavily positive.
Rabbit 2,	30 min. 5 hrs. 12 hrs. 24 hrs. 36 hrs. 48 hrs. 72 hrs. 96 hrs. 120 hrs. 144 hrs. 168 hrs. 192 hrs.
weight 1,720 gm.	30 1 80 50 30 30 25 0 10 50 200 ×
Autopsy.	216 hrs., rabbit died. Pericarditis, and extensive cellulitis over the abdomen. Smears from these lesions were heavily loaded with streptococci.

such an extent that the bacteria were able to produce a general infection. Or, on the other hand, the life within the tissues of the host increased the infectivity of the bacteria and they were able to overcome the opposition offered by the rabbit.

Experiment 4.—This experiment was made one month after experiment 3. The bacteria had been kept on artificial media during this time. The quantities of culture used and other points of technique were the same as in the preceding experiments. Three rabbits are given in table IV to represent the nature of infections obtained at this time.

In experiment 4 the bacteria were almost devoid of infecting power and they behaved very much as saprophytes; they rapidly disappeared from the circulation and were not able to reappear. The rabbits showed no signs of disease and remained in perfect condition.

PNEUMOCOCCUS INFECTIONS.

A number of experiments were carried out with the pneumococcus similar to those just described with the streptococcus. Depending upon the virulence and the number of bacteria injected, a pneumococcic infection in the rabbit may take any one of the three courses described in the streptococcic infections. The initial decrease in the number of bacteria in the blood may not be as complete as with the streptococcus, especially if a very large number of bacteria are injected. Pneumococci usually begin to reappear in the blood earlier than streptococci. The infections are more acute and severe. The types of infection are even more defined than with the streptococcus. The acute infections last from two to three days. In from two to three hours the bacteria have largely disappeared from the blood. Cultures taken at five hours usually show an increase in the number of colonies and this reaches a heavy septicemia in from eighteen to twenty-four hours. The animals die within forty-eight to seventy-two hours. Rabbit 1 in table V is an example of this type of infection. If the bacteria have lost in virulence or if a smaller number is injected, a chronic infection occurs. Rabbit 2 of table V represents this class typically. The bacteria disappear from the blood more rapidly and the reappearance is delayed and the increase is slight and is followed by a second decrease. The rabbits usually have a low septicemia for several days. This is followed by a rise and the animal dies within one to two

TABLE IV.

Animal.	Time of bleeding and number of colonies.							
Rabbit 1, weight 1,380 gm.	30 min.	2 hrs.	20 hrs.	26 hrs.	44 hrs.	66 hrs.	72 hrs.	The bacteria never reappeared in the blood and the rabbit was in perfect condition 1 mo. later.
	75	4	3	5	20	0	0	
Rabbit 2, weight 1,620 gm.	10 min.	4 hrs.	20 hrs.	The blood remained sterile and the rabbit was in good condition after 1 mo.				
	180	0	0					
Rabbit 3, weight 1,200 gm.	3 hrs.	20 hrs.	44 hrs.	64 hrs.	112 hrs.	Bacteria never reappeared in the blood, and the rabbit continued well.		
	7	0	12	0	0			

days. At autopsy severe local lesions are always found. The localization may be in the pericardium, pleura, peritoneum, or in the subcutaneous tissues of the abdomen. In one rabbit localization occurred in the kidneys. Any two or more of these places may be involved in the same animal, but many times only one is affected. The bacteria accumulate in great quantities in these localities; when present, septicemia almost invariably precedes death. In the third type the rabbit masters the situation within a few hours; the bacteria disappear from the blood permanently and the animal continues in perfect condition. Rabbit 3 in table V falls in this class.

SUMMARY.

When rabbits are injected intravenously with a quantity of virulent streptococci or pneumococci sufficient to cause death within two to four days the septicemia takes a definite course with slight variations. The bacteria rapidly decrease in number from the time of the injection to from two to four hours, at which time the blood is sterile or contains only a few bacteria. Within five to six hours the bacteria reappear in the blood and steadily increase until the death of the animal. If the bacteria are less virulent, the same quantity of culture causes a chronic type of infection. The same initial decrease in the number of bacteria occurs. The reëntrance into the blood is somewhat delayed, the septicemia does not reach the height obtained in the acute cases, and a second fall occurs within the course of a few hours. These rabbits show a low blood invasion or a sterile blood culture for several days. During this time they become emaciated to a marked degree. Then the low septicemia rapidly rises or the rabbit with a sterile culture develops a severe septicemia within a few hours and death takes place from a few hours to two days thereafter. In this type of infection local lesions, pericarditis, pleurisy, peritonitis, etc., are usually found. In the infections which run an acute course no gross lesions are found. If the bacteria are still less virulent they never reënter the blood after the initial disappearance and the rabbits remain in good condition. In order to obtain uniform results, the quantity of bacteria injected must not be so large that the bacterial substances

TABLE V.

Animal.	Time of bleeding and number of colonies.										
Rabbit 1, weight 1,450 gm.	30 min.	3½ hrs.	22 hrs.	27 hrs.	In 44 hrs. rabbit was found dead.						
	150	0	×	×							
Rabbit 2, weight 1,700 gm.	5 min.	30 min.	1½ hrs.	3 hrs.	4½ hrs.	7 hrs.	11 hrs.	21 hrs.	25 hrs.	28 hrs.	48 hrs.
	350	3	0	0	3	15	1	100	350	200	125
	72 hrs.	96 hrs.	Dead 120 hrs. Autopsy showed pleurisy, pericarditis, and cellulitis over the abdomen. Films from these places were heavily loaded with pneumococci.								
	100	200									
Rabbit 3, weight 1,620 gm.	10 min.	4 hrs.	20 hrs.	The blood remained sterile and the rabbit fully recovered.							
	180	0	0								

carried in are sufficient to cause an intoxication of the animal. If the quantity of bacteria injected is below this point the course of the infection depends largely upon the virulence of the infecting organisms. Yet variations in the natural resistance of individual animals may be sufficient to cause quite marked irregularities in the course of the infection. Pneumococci can be standardized so as to produce a particular type of infection more easily than streptococci. In general infections such as those produced by streptococci and pneumococci the number of the bacteria present in the circulating blood at a given time supplies accurate and delicate information regarding the severity of the disease. When the object is to determine the degree of virulence of bacteria, or of the efficiency of an experimental therapeutic method, the mere physical condition and mere death of the inoculated animals are not sufficient and satisfactory guides to the desired information. The death of the inoculated animal and the recovery of the infecting bacteria at autopsy do not give complete information concerning the intensity and course of the infection occurring during life. A large number of bacteria found in the blood and tissues at autopsy do not necessarily prove the existence of a heavy infection before the onset of the death agony, since it is a well known fact that bacteria multiply with enormous rapidity, once the natural resistance of the animal has been overcome. Therefore, if merely the life and death of the animal and autopsy findings must serve as our only guides, we shall lose much incidental information, perhaps of fundamental value. This may be especially true as regards the search for curative substances. Again, the individual animals of the same species, age, and apparently of identical physical condition react to the aggressive force of the infecting organisms variously. This fact is readily found out by the injection of a series of rabbits with lethal quantities of bacteria per body-weight, and by making tests at various periods before death results, which, in the case of streptococci, ranges from one to six days. Consequently a method which enables the determination of the degree and progress of the infection at any desired period is of obvious advantage.

LOCALIZATION OF THE VIRUS AND PATHOGENESIS OF EPIDEMIC POLIOMYELITIS.*

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Since epidemic poliomyelitis is an affection chiefly of the central nervous tissues, it may be supposed that the nervous organs possess a special affinity for its microbic cause.[1] Once the virus of the disease has gained access to, and multiplied within, the nervous tissues, it survives there, apparently, longer than it does in other organs in which, under ordinary conditions, it occurs far less regularly. It has been shown by experiment that the poliomyelitic virus readily reaches the central nervous system when it is brought into relation with the peripheral nerves. It is in this manner that infection is induced when the virus is brought into contact with the nasal mucosa, sciatic nerves, and probably also when it is injected into the subcutaneous tissues and peritoneal cavity.[2] Thus deposited, the virus ascends by way of the nerves to the olfactory lobes of the brain or to the spinal cord and intervertebral ganglia. It is to be assumed that in the case of the spinal nerves the ascent is by way of the afferent or sensory fibers; in the case of the nasal membrane, along the olfactory fibers. Hence the virus is carried both by nerves of common and of special sensation. In these instances the virus is brought into relation, not with special end organs, but with nerve fibrils, along which it travels. It appears, however, that the virus may enter the nerves by way of specialized end organs, which are themselves not appreciably injured by it.

INTRAOCULAR INOCULATION.

The demonstration that the virus of poliomyelitis may penetrate the uninjured sensory end organs has been made in connection with the optic nerve. An emulsion of the spinal cord carrying the active

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[1] Flexner, S., *Jour. Am. Med. Assn.*, 1910, lv, 1105.

[2] Flexner, S., and Amoss, H. L., *Jour. Exper. Med.*, 1914, xix, 411.

virus and free from bacterial contamination can be injected into the vitreous chamber of the eye without causing appreciable inflammation. A small quantity of the vitreous humor is aspirated, under ether anesthesia, by means of a tuberculin syringe carrying a very fine needle, and replaced by an emulsion of the virus. The cloudiness thus caused tends to disappear, and the vitreous body to return to normal. After a variable incubation period, symptoms of experimental poliomyelitis appear, paralysis develops, and the pathological findings are characteristic of the disease.

Experiment 1.—Macacus rhesus. May 29. Withdrew under ether anesthesia as stated 0.1 c.c. vitreous humor by puncture of sclera and introduced 0.2 c.c. emulsion of active virus. June 7. Excitable. June 9. Weakness of arms. June 10. Ataxic. June 12. Paralysis of right arm; weakness of left leg; both eyes normal. June 15. Arms and legs weak. From this date the condition remained stationary until June 22, when death occurred from diarrhea.

The autopsy revealed old and recent dysenteric lesions of the large intestine. No visible changes were observed in the spinal cord or eyes. However, microscopical examination of the spinal cord, medulla, and intervertebral ganglia shows typical lesions of poliomyelitis. The retina of the inoculated eye appears normal.

Experiment 2.—Macacus rhesus. June 12. Withdrew, in the manner of experiment 1, 0.1 c.c. vitreous humor from the left eye and injected 0.2 c.c. paper filtrate of an emulsion of virus. The cloudiness of the vitreous humor did not entirely clear up. June 19. Excitable. June 22. Tremor of head; weakness of left leg; ataxia. June 23. Paralysis of left arm; weakness of right arm; double ptosis and slight left facial paralysis; weakness of left leg. June 24. Prostrate; etherized.

The spinal cord, medulla, and intervertebral ganglia present typical lesions of poliomyelitis. The ophthalmic ganglion on the inoculated side shows also a diffuse cellular infiltration.

The two experiments given confirm the supposed affinity which the poliomyelitic virus possesses for nervous tissues and they indicate also that the virus is capable of penetrating highly specialized end organs in order to reach peripheral nerves, along which it penetrates to the central nervous system. Incidentally it shows that the virus may pass from peripheral nerves into the adjacent ophthalmic ganglion, but whether directly from the eye, or indirectly after infection of the central nervous organs as occurs with the Gasserian ganglia, does not appear.[3]

[3] Landsteiner, K., and Levaditi, C. (*Compt. rend. Soc. de biol.*, 1909, lxvii, 787) succeeded in one instance in producing paralysis by inoculating the virus into the anterior chamber of the eye.

DISTRIBUTION OF THE VIRUS AFTER INTRAVENOUS INOCULATION.

It is established that experimental poliomyelitis may be caused with more or less regularity by insuring that the virus reaches the central nervous organs by way of the peripheral nerves. When the virus is brought directly into relation with the central nervous system by intracerebral and intraspinous injections the most constant results are secured. Probably the less constant effects which follow injection of the virus into the peripheral nerves result from the fact that the greater the distance the virus is compelled to travel along nerves, the more chances there are for miscarriage of infection, either by reason of too great dilution, or failure of the virus to reach the central organs at all.

In all these instances the affinity of the nervous organs for the virus may be exerted directly, since the virus is brought either immediately into relation with the nervous tissues, or reaches them directly through lymphatic communication. When the virus is brought to the nervous organs by means of the blood, it is at first separated from the tissues themselves by the vessels and other structures interposed between the blood itself and the nervous tissue. For this reason it has been observed that, while small or even infinitesimal doses of the virus suffice to induce quite constant infection by the intranervous mode of inoculation, large quantities of the virus produce only occasional and inconstant infection, when injected directly into the blood.

The cause of this discrepancy has already been traced to an apparent inability of the virus to enter directly the substance of the brain and spinal cord from the blood.[4] In order to reach these organs, the virus must, it appears, leave the blood and pass into the cerebrospinal fluid, with which it reaches the interstices of the tissues. Since the cerebrospinal liquid is a product of the activity of the choroid plexus, it has been assumed that the virus must first penetrate that structure. Experiments have been performed in order to study this phase of the subject more closely.

Infectivity of Organs.—When the virus is injected directly into the blood it is quickly distributed throughout the circulatory system

[4] Flexner and Amoss, *loc. cit.*

in the manner in which other microörganisms are distributed. In due time it may be assumed that the virus is deposited in certain organs, since experiment has shown that it does not remain long in the circulating blood.[5] Hence it is readily possible to ascertain the distribution of the virus by sacrificing at intervals the infected animals, and inoculating emulsions of the organs themselves. In this manner it can be determined whether the virus is distributed mechanically, or according to the affinity which the several organs display toward it.

The next experiments to be described have been devised to answer this question.

Experiment 3.—Macacus rhesus. May 4. 250 c.c. of a centrifugalized suspension of the spinal cord and medulla containing the active virus were injected into one of the superficial veins of the leg. Three days later, on May 7, the animal was etherized and the spinal cord, medulla, crura cerebri, and intervertebral ganglia were removed aseptically. These were made into 5 per cent. suspensions which were injected intracerebrally into three *Macacus rhesus* monkeys, A, B, and C.

Monkey A.—May 7. 2 c.c. of emulsion of intervertebral ganglia injected. May 10. Excitable. May 12. Right arm weak; left arm paralyzed. May 13. Prostrate; etherized. Typical poliomyelitis.

Monkey B.—May 7. Injected 2 c.c. of emulsion of spinal cord and medulla. This animal developed no symptoms and remained well indefinitely.

Monkey C.—May 7. Injected 2 c.c. of emulsion of crura cerebri. No symptoms of poliomyelitis developed and the animal remained well indefinitely.

Experiment 4.—Macacus rhesus. Apr. 16. 240 c.c. of centrifugalized virus were injected intravenously. Four days later, Apr. 20, the animal was etherized. There were removed aseptically for inoculation: spleen, bone marrow, kidneys, spinal cord and medulla, which were made into 5 per cent. emulsions and injected intracerebrally into *Macacus rhesus* monkeys, D, E, F, and G.

Monkey D.—Apr. 21. Received 2 c.c. of the suspension made from portions of spinal cord and medulla. No symptoms of poliomyelitis developed, and the animal remained normal.

Monkey E.—Apr. 21. Received 2 c.c. of a suspension of portions of each kidney. No symptoms of poliomyelitis developed, and the animal remained normal.

Monkey F.—Apr. 21. Received 2 c.c. of a suspension of the spleen. Apr. 21. Excitable; ataxic. Apr. 25. Both legs and right arm paralyzed. Apr. 27. Died. Typical poliomyelitis.

Monkey G.—Apr. 21. Received 2 c.c. of suspension of bone marrow of both femurs. Apr. 24. Excitable. Apr. 29. Tremor; weakness of neck. Apr. 30. Prostrate; etherized. Typical poliomyelitis.

[5] Clark, P. F., Fraser, F. R., and Amoss, H. L., *Jour. Exper. Med.*, 1914, xix, 223.

Experiment 5.—Macacus rhesus. Mar. 31. Intravenous injection of 250 c.c. of centrifugalized virus. Five days later, Apr. 5, etherized and portion of spleen, bone marrow, spinal cord and medulla were removed aseptically and made into 5 per cent. emulsions and injected intracerebrally into *Macacus rhesus* monkeys, H, I, and J.

Monkey H.—Apr. 5. Injected 2 c.c. of suspension of spinal cord and medulla. No symptoms developed and the animal remained normal.

Monkey I.—Apr. 5. Injected 3 c.c. of the suspension of the spleen. Apr. 8 Excitable. Apr. 11. Ataxic. Apr. 12. Tremor; prostrate. Apr. 13. Died. Typical poliomyelitis.

Monkey J.—Apr. 5. Injected 3 c.c. of suspension of bone marrow from both femurs. Apr. 10. Excitable; ataxic; weakness of left leg. Apr. 17. Legs and left arm paralyzed; prostrate; etherized. Typical poliomyelitis.

The sections of the central nervous organs show not only marked lesions of poliomyelitis, but those of the fourth ventricle reveal an infiltration of the choroid plexus with mononuclear cells.

This series of experiments is consistent in exhibiting that, in spite of the essential affinity which the spinal cord and brain exhibit for the poliomyelitic virus, they are, nevertheless, unable to remove it directly from the blood, prior to some change taking place in the structures (vascular or secretory) that preside over the production of the cerebrospinal fluid; while the spleen and bone marrow, but not the kidney, readily remove it from this source. The fact that the intervertebral ganglia are capable of readily removing part of the virus from the blood shows, first that their relation to the blood vessels differs from that of the brain and spinal cord, and second explains the constant and early involvement of these structures in the poliomyelitic process. The ganglia, therefore, appear capable of obtaining the virus from two sources, namely, directly from the blood, and indirectly from the cerebrospinal fluid.

Moreover, the poliomyelitic virus may be retained alive in the body for a considerable period of time without gaining access to the interior of the central nervous organs.

Experiment 6.—Macacus rhesus. Apr. 18. Injected intravenously 250 c.c. of centrifugalized virus. No symptoms of poliomyelitis developed and seventeen days later, May 5, the animal was etherized. 5 per cent. suspensions of spleen and central nervous tissues were prepared and inoculated intracerebrally in monkeys K and L. Microscopic examination of sections of the spinal cord, medulla, and intervertebral ganglia prove them to be free from lesions of poliomyelitis.

Monkey K.—May 6. 2 c.c. of emulsion of medulla and spinal cord injected. No symptoms developed, and the animal remained normal.

Monkey L.—May 6. 2 c.c. of suspension of spleen injected. May 12. Excitable; ataxic. May 16. Arms weak; etherized. Poliomyelitis.

This experiment indicates, first that the virus of poliomyelitis is capable of surviving for a considerable period in the interior of the body, without inducing an infection of the central nervous system; and next that this long sojourn is not without effect on the quality of the virus, which would appear to have been weakened by the action of the spleen.

Effect of Aseptic Meningitis.—The permeability of the meninges for the contents of the blood is increased by inflammation of those structures. The introduction of sterile alien blood serum into the subarachnoid spaces causes an aseptic inflammation of mild degree that reaches its maximum in twenty-four hours, and then subsides. The inflammation is marked by emigration into the pia-arachnoid, cerebral ventricles, and choroid plexus of polymorphonuclear leucocytes chiefly, and by the escape of plasma. Neither the ependymal epithelium nor the perivascular lymphatics show appreciable change.

Experiments were conducted to ascertain the effect of this inflammation on the penetration of the virus of poliomyelitis into the central nervous organs.

Experiment 7.—Control. *Macacus rhesus.* Apr. 23. Injected intravenously 25 c.c. of centrifugalized suspension of spinal cord and medulla of paralyzed monkey. No symptoms developed and the animal remained normal.

Experiment 8.—Control. *Macacus rhesus.* Apr. 23. 50 c.c. of the centrifugalized suspension of virus used in the previous experiment were injected intravenously. The animal remained normal.

Experiment 9.—*Macacus rhesus.* Apr. 22. Injected intraspinously 3 c.c. of 40 per cent. inactivated horse serum. Apr. 23. Lumbar puncture; 0.5 c.c. of turbid fluid containing large numbers of white corpuscles was obtained. Injected intravenously 25 c.c. of centrifugalized suspension of virus as in experiments 7 and 8. Apr. 26. Excitable; legs weak. Apr. 30. Arms also weak; etherized.

Microscopic examination of the medulla, spinal cord, and intervertebral ganglia reveals pronounced lesions of poliomyelitis. While vascular lesions are everywhere pronounced, lesions of the nerve cells, interstitial substance, and meninges are also marked. The choroid plexus of the fourth and lateral ventricles is included in the sections. The plexus of the fourth ventricle shows definite infiltration with mononuclear (lymphoid) cells; the plexus of the lateral ventricle is less infiltrated. The blood vessels in the floor of the fourth ventricle are heavily infiltrated, while the deeper vessels are less affected, and those beneath the lateral ventricle are unaffected.

Experiment 10.—Macacus rhesus. May 5. Injected intraspinously 3 c.c. of 40 per cent. inactivated horse serum. May 6. Injected intravenously 25 c.c. of centrifugalized suspension of active virus. May 9. Excitable. May 13. Right arm paralyzed. May 14. Died.

The microscopic examination of sections of the medulla, spinal cord, and ganglia shows poliomyelitic lesions of moderate degree. The most pronounced lesions occur in the floor of the fourth ventricle. Vascular lesions are nowhere severe.

The foregoing observations raise anew the question as to the path traversed by the virus from the blood to the cerebrospinal fluid, and thence to the nervous tissue. Since the cerebrospinal liquid is the product of the secretory activity of the choroid plexus, it has been assumed that the impermeability of the plexus for most foreign products results in the exclusion also of microörganisms, as long as the secreting structures remain intact. Apparently such moderate quantities of the poliomyelitic virus as are contained in twenty-five to fifty cubic centimeters of a clear suspension of the spinal cord and medulla, taken from a paralyzed monkey, may be insufficient to inflict the necessary damage upon the choroid plexus, while still larger quantities may suffice to accomplish this. When, however, the superficial structures of the nervous organs, such as the meninges and choroid plexus, are put into a state of mild chemical inflammation, their permeability is increased, so that what was before an inadequate quantity of virus is now rendered sufficient to cause poliomyelitic infection.

Another fact has emerged from these experiments: while the blood vessels of the spinal cord and brain may show no unusual degree of pathological alterations, definite lesions of an infiltrative nature may appear in the choroid plexus itself. Before, however, a decision is reached as to the relation of the histological lesions to the escape of the virus from the blood, it is desirable to determine the nature of the lesions in animals that have developed paralysis from large unaided injections of the virus, administered intravenously.

Lesions Caused by Intravenous Injections.—While rhesus monkeys show almost no difference in susceptibility to the action of the poliomyelitic virus when introduced directly into the brain, they exhibit distinct differences when injections are made into the peripheral

parts of the nervous system, or into the blood. Moreover, the quality of the virus itself is brought out by the site of inoculation, since a specimen that is of less than maximal activity may be infectious even in minute doses, when introduced into the brain, and either not active at all, or slightly infectious when injected into the blood, or even into the sciatic nerves. As the experiments that follow show, the quantity of the centrifugalized suspension carrying the virus required to cause infection by the unaided blood route usually exceeds fifty cubic centimeters, while even 200 or more sometimes fail, although a few tenths of a cubic centimeter of the same virus succeed when introduced into the brain.

Before describing the lesions present in the spinal cord, brain, and intervertebral ganglia, caused by intravenous injections, it is desirable to present briefly the nature of the lesions of experimental poliomyelitis such as result from other modes of inoculation. The several lesions may be considered as they affect first the meninges, second the spinal cord, third the medulla and pons, fourth the cerebrum, and fifth the ganglia.[6]

The meninges of the cord and medulla show, as a rule, mononuclear cellular infiltration most pronounced adjacent to, or surrounding, the blood vessels which enter the fissures of the cord and are present in the floor of the fourth ventricle. The general infiltration of the pia-arachnoid is interstitial and as a rule not heavy, while the invasion about the vessels within the perivascular lymphatics is usually heavy, and sometimes is nodular. The spinal cord presents lesions most pronounced in the anterior gray matter, less marked in the posterior gray matter, and least present in the white matter. They are perivascular, interstitial, and parenchymatous. The vascular lesions, which are often pronounced, extend inward from the meninges; the interstitial ones are associated with the presence of mononuclear, to a less extent of polynuclear cells, rarely of red corpuscles, and commonly of serum. Actual necrosis of the ground substance arises, but is uncommon on a large scale. The anterior gray matter is rarely wholly destroyed at certain levels. The interstitial lesions can, in some instances, be traced outwards, directly from affected vessels. The lesions of the parenchyma consist of degeneration and necrosis of ganglion cells, occurring chiefly but not exclusively in the anterior gray matter. The necrotic cells are commonly invaded by phagocytes, the so called neurophages. It is not usual for definite relation to be obvious between the altered blood vessels and the affected interstitial substance or parenchyma. The lesions of the medulla resemble those of the spinal cord, except as they are modified by differences in structure. The vessels most infiltrated are those present im-

[6] Flexner, S., and Lewis, P. A., *Jour. Exper. Med.*, 1910, xii, 227.

mediately beneath the fourth ventricle; the deeper lying vessels tend to be less affected, and the very small branches throughout the part are involved inconstantly. The focal interstitial lesions tend to be smaller than those of the cord. No definite relation can, as a rule, be made out between the vascular and interstitial changes. Because of the smaller size and less uniform distribution of nerve cells, the parenchymatous lesions are less conspicuous; they are, however, essentially identical with those of the cord. Lesions similar to those in the medulla occur in the pons and crura cerebri, but less frequently. The cerebrum is affected far less constantly than other parts of the nervous system. When present, the lesions are perivascular and focal interstitial. The cerebral meninges, as a rule, escape affection. The choroid plexus of the lateral and fourth ventricles has not been studied in all instances. When the virus has been introduced into the brain, cerebrospinal fluid, or nerves, the rule appears to be that the plexus escapes. However, exceptions to this rule occur, in which case lesions similar to those to be described as occurring after intravenous injection of the virus may arise (compare experiment 5, monkey J). The intervertebral ganglia are invariably affected. The lesions are of two main kinds, interstitial and parenchymatous, and are always focal. The cellular invasion proceeds from two sources; the pial investment and the blood vessels. In the former, direct extension may take place from the spinal meninges, or extension may occur by way of the connective tissue of the nerve roots. In the latter, extension seems to proceed from the blood vessels. It remains, however, to state that the involvement of the blood vessels may not arise through the general blood, but through inclusion of the vessels in the infiltrative process within the septa of the nerve roots. In rare instances the blood vessels present, as compared with other parts of the ganglia, an unusual degree of surrounding infiltration. The ganglionic nerve cells are destroyed in two ways: first, they are obliterated by focal accretions of mononuclear cells, and, second, by necrosis and neurophagocytosis, in the same manner as in the corresponding condition in the spinal cord.

With this description before us, we may now proceed to describe the lesions which arise as a result of infection by the intravenous mode of inoculation.

Experiment 11.—Macacus rhesus. Nov. 14. 100 c.c. of a Berkefeld filtrate of the virus were injected into the right saphenous vein. Nov. 24. Excitable; paralysis of the left leg and left side of the face. Nov. 25. Paralysis of arms and legs; weakness of back. Nov. 26. Prostrate. Dec. 1. Died.

The spinal cord, medulla, and ganglia show pronounced lesions. Those of the spinal cord affect the blood vessels, interstitial tissue, and nerve cells. They are not distinctive, and do not differ from the usual lesions. This fact is also true of the lesions in the medulla, which are most pronounced in the blood vessels in the floor of the fourth ventricle, and of the ganglia, where they are wide-spread.

Experiment 12.—Macacus rhesus. Nov. 7. Injected intravenously 54 c.c. of a Berkefeld filtrate of the virus. At the same time, under ether anesthesia, 5 c.c. of salt solution were introduced into the left cerebral hemisphere. Nov. 16. Excitable; ataxic. Nov. 17. Arms paralyzed; back weak. Nov. 18. Prostrate. Nov. 19. Etherized.

The lesions of the spinal cord, medulla, ganglia, and cerebrum are pronounced. Not only are the usual lesions present, but in addition severe affection of the blood vessels in the cord, medulla, and cerebrum occurs, from which infiltrations extend into the substance of the nervous tissues. The cerebral meninges at the site of the injection of salt solution also show infiltration.

Experiment 13.—Macacus rhesus. Jan. 30. 250 c.c. of centrifugalized suspension injected intravenously. Feb. 14. Excitable. Feb. 16. Weakness of legs. Feb. 17. Paralysis of arms. Feb. 18. Weakness of back. Feb. 19. Died.

The lesions are typical of poliomyelitis. The meninges of the spinal cord are diffusely infiltrated. The vascular lesions are moderate. There is widespread degeneration of the nerve cells, but none of the lesions are distinctive, or differ from those usually occurring.

Experiment 14.—Macacus rhesus. Oct. 30. Intravenous injection of 180 c.c. of centrifugalized virus. Nov. 9. The animal shows weakness and disturbance of vision. During the night it died.

The medulla, spinal cord, and ganglia are the seat of marked lesions of poliomyelitis. The vascular infiltration is heavy but the usual larger vessels only are affected. The cerebrum is devoid of lesions, while the crura cerebri are the seat of interstitial, but not of striking vascular lesions.

Experiment 15.—Macacus rhesus. Feb. 28. Intravenous injection of 250 c.c. of centrifugalized virus. Mar. 4. Excitable. Mar. 5. Arms and legs weak; ataxic. Mar. 6. Died.

The lesions of the spinal cord are perivascular, interstitial, and meningeal; of the ganglia, perivascular, with extension into the nerve roots. The medulla shows a high degree of affection of the blood vessels. The cerebrum has escaped, but the choroid plexus of the lateral ventricle, but not of the fourth ventricle, shows edema and perivascular cellular infiltration. The ependymal cells appear normal.

Experiment 16.—Macacus rhesus. Apr. 16. Intravenous injection of 250 c.c. of centrifugalized virus. During the injection a needle was kept in the lumbar spinal canal. Apr. 21. In the morning the arms and back were paralyzed. In the afternoon death occurred.

The lesions in this instance are very pronounced. The blood vessels within the spinal cord, medulla, and pons show wide involvement, while the interstitial tissue and nerve cells are affected only moderately. The cerebrum is devoid of lesions, while the choroid plexus of the lateral ventricles contains a slight accumulation of mononuclear cells about the blood vessels. The plexus of the fourth ventricle appears normal.

Experiment 17.—Macacus rhesus. Apr. 16. 240 c.c. of centrifugalized virus injected intravenously. Apr. 20. Lumbar puncture yielded a fluid containing an excess of white corpuscles. Apr. 21. Excitable; no paralysis; etherized.

The spinal cord, medulla, and pons show early vascular, but no other lesions. The ganglia, however, contain focal cellular infiltrations of small size, and a small number of single necrotic nerve cells. No changes were detected in the choroid plexus.

Experiment 18.—Macacus rhesus. Apr. 16. 240 c.c. of centrifugalized virus injected intravenously. Apr. 20. No symptoms appeared; etherized.

No lesions were detected in the spinal cord, medulla, or choroid plexus, while the ganglia show early infiltrative lesions about the blood vessels, and a few instances of necrosis of single nerve cells.

We may consider this series of experiments according as the lesions affect the nervous tissues proper, or as they affect the choroid plexus.

Within the nervous tissues proper, the lesions are, at times, precisely similar to, and indistinguishable from, those produced by intraneural modes of inoculation. However, in certain instances, the lesions present not only resemble those caused by the intraneural modes of inoculation, but differ from them in the extent and degree to which the blood vessels, and those especially in the medulla and pons, are affected. While the degree of perivascular infiltration does not afford a basis of discrimination, a sharp distinction may be drawn between the usual degree of vascular involvement, and the unusual extent in which it occurred in several cases of intravenous injection. What is especially impressive in the latter instances is the diffuse participation of small vessels, down to those of capillary size, in the process and the extension of the infiltrative process from them to the surrounding nervous tissues. Vessels so greatly altered as those under consideration may be considered as contributing to the permeation of the virus from the blood into the tissues. The early lesions of the intervertebral ganglia should be emphasized in this place since they antedate those of the spinal cord and medulla, and extend apparently from vascular lesions.

What appear, however, to be especially important are the changes detected in the choroid plexus, in which infiltrative lesions have hitherto not been observed. That definite lesions of the plexus may occur is clearly indicated by the experiments. As yet no evidence has been obtained of morphological alterations in the ependymal cells, but merely in the blood vessels beneath them. That the secretory functions of the plexus are altered in the direction of greater permeability may be safely assumed, from which it follows that the experiments indicate that when the poliomyelitic infection is induced by the intravenous injection of the virus, there arise, not only the common lesions of poliomyelitis, but also certain additional lesions of the blood vessels and choroid plexus which are of peculiar and distinctive nature.

Since the precise mode of infection in human cases of poliomyelitis may be regarded still as an open question, this criterion of a blood invasion may prove of assistance in the solution of the problem. So far as can be judged from the study of the tissues from several human cases, a corresponding wide-spread vascular involvement to that arising in the experiments would seem not to have occurred.

INTRASPINOUS SERUM PROTECTION.

The data presented confirm and extend the observations already made concerning the passage of the poliomyelitic virus in transit to the central nervous organs from the blood to the cerebrospinal fluid. Since the virus is known to pass successively over several days into the cerebrospinal fluid,[7] in which it seems not to accumulate, but from which it is transferred to the nervous tissues, it was thought that the introduction of a potent immune serum into the meninges at intervals over a number of days would suffice to neutralize the translated virus and thus prevent infection. For this purpose immune serum was available from several monkeys which had recovered from poliomyelitis and had been subsequently reinforced by large subcutaneous injections of the virus.

Earlier experiments had shown that the intraspinous injection of an immune serum is effective under circumstances in which the intravenous injection is not, in delaying or preventing poliomyelitic infection in the monkey. For the next series of experiments it was necessary at the outset to insure that the intravenous injections of the virus would alone induce paralysis, which was accomplished by employing the device of setting up an aseptic meningitis with horse serum, in the animals about to be inoculated.

SERUM AFTER INTRAVENOUS INOCULATION.

Experiment 19.—Macacus rhesus. May 26. Intraspinous injection of 3 c.c. of inactivated 40 per cent. horse serum. May 27. Intravenous injection of 50 c.c. of centrifugalized virus, followed immediately afterwards by an intraspinous injection of 3 c.c. of normal monkey serum. The normal serum was injected intraspinously on May 28, 29, and 30, and, after a two days' interval, on June 2, 3, and 4. June 3. Excitable. June 9. Paralysis of arms and back. June 10. Prostrate; etherized. Typical poliomyelitis.

[7] Flexner and Amoss, *loc. cit.*

Experiment 20.—Macacus rhesus. May 29. Intraspinous injection of 3 c.c. of inactivated 40 per cent. horse serum. May 30. Intravenous injection of 50 c.c. of centrifugalized virus, followed immediately afterward by an intraspinous injection of 3 c.c. of immune monkey serum. The immune serum injections were repeated May 31, June 1 and 2, and, after a two days' interval, on June 5, 6, and 7. No symptoms developed, and the animal remained normal.

Since these experiments show that the virus may be neutralized by an immune serum in process of passage by way of the cerebrospinal fluid to the nervous tissues, it seemed desirable to ascertain whether a similar neutralization could be affected in a case in which the virus was introduced directly into the meninges by means of lumbar puncture.

SERUM AFTER INTRASPINOUS INOCULATION.

Experiment 21.—Macacus rhesus. May 27. Intraspinous injection of 1 c.c. of emulsion of virus. Two hours later, intraspinous injection of 3 c.c. of normal monkey serum. The injection of normal serum was repeated on May 28, 29, and 30, and, after a two days' interval, on June 2, 3, and 4. June 3. Excitable. June 7. Ataxia; arms and neck weak. June 8. A.M. Arms and back paralyzed; legs weak. P.M. Died. Typical poliomyelitis.

Experiment 22.—Macacus rhesus. May 27. Intraspinous injection of 1 c.c. of emulsion of virus. Two hours later, injected 3 c.c. of immune monkey serum intraspinously. The immune serum injections were repeated on May 28, 29, and 30, and, after a two days' interval, on June 2, 3, and 4. No symptoms developed and the animal remained well.

The preceding experiments show unmistakably that by introducing an immune serum into the subarachnoid spaces, the poliomyelitic virus is capable of being neutralized within the cerebrospinal fluid into which it is directly introduced, or to which it passes in transit from the blood to the nervous tissues. Probably the neutralization in the latter instance is effected at successive stages in process of transfer of the virus to the central nervous organs. Normal serum lacks this power of neutralization.

It may be considered as highly probable that the neutralization is accomplished before any quantity of the virus becomes attached to the nervous tissues themselves. Earlier experiments had shown that when such minute amounts of the virus as one fiftieth to one tenth of a cubic centimeter are inoculated intracerebrally, neutralization is either wholly impossible to accomplish, or is accomplished with very great difficulty even by intraspinous injections of immune

serum.[8] Hence the experiments described carry a step further the demonstration that the virus introduced into the blood passes by way of the cerebrospinal fluid to the substance of the nervous tissues in those instances in which paralysis results.

RELATION OF CARMIN TO THE CHOROID PLEXUS AND PERIVASCULAR LYMPHATICS.

That the virus of poliomyelitis is capable, in some instances, of passing from the blood to the cerebrospinal fluid may be considered as demonstrated. This passage takes place probably by way of the choroid plexus and possibly also, to some extent, through the blood vessels in the meninges as well as in the substance of the nervous tissues. Although certain lesions have been detected in the choroid plexus, no morphological alterations have been discovered in the ependymal cells themselves. Hence the question arose whether by the use of pigments the ependymal cells in certain pathological states, including poliomyelitis, might be shown to react in a manner supplying ocular evidences of a disturbance of function.

Carmin is a non-toxic pigment which can be sterilized and suspended in a fine state of subdivision. In this form its introduction into the cerebral ventricles and subarachnoid spaces causes no discomfort in monkeys. Its presence in the meninges and ventricles is followed by an inflammation and rich cellular exudation. The emigrated cells are polynuclear chiefly, but mononuclear cells which take up pigment granules emigrate also. The effects of the carmin injections were studied in normal monkeys, and in monkeys in which an aseptic inflammation had been set up twenty-four hours earlier by means of horse serum, or in which poliomyelitis had been induced by intracerebral inoculation of the virus.

A suspension of the pigment was made in 20 per cent. glycerin, and ammonia was added until solution was complete. This solution was autoclaved and immediately before use was slowly neutralized by the repeated addition of small amounts of sterile 2 N hydrochloric acid, until litmus paper indicated change of reaction. The injections were made under ether anesthesia into the lateral ven-

[8] Flexner and Lewis, *Jour. Am. Med. Assn.*, 1910, liv, 1780; 1910, lv, 662.

tricle, the volume of fluid injected being determined by a needle in the lumbar meninges, from which the colored solution was allowed to flow before the injection was stopped.

Within twenty-four hours the pigment is distributed over the surfaces of the spinal cord and brain, and within the cerebral ventricles. The base of the brain is deeply and uniformly pigmented. The intervertebral ganglia are either unaffected or mottled with pigment. The nerve roots are visibly pigmented. The choroid plexus appears a vivid red color.

Normal monkeys etherized respectively twenty hours and five days after the injection of the pigment show the inflammatory reaction of the meninges and ventricles mentioned. Interest centers especially in the relation of the pigment to the ependymal cells, choroid plexus, perivascular lymphatics, and intervertebral ganglia. The differences in this respect between the twenty-hour and the five-day specimens are inconspicuous and unimportant.

The pigment appears in two states of division, namely, as excessively minute particles, smaller than many bacteria, and as coarser grains. The latter are contained largely in the mononuclear cells of the inflammatory exudate. The very fine particles have been taken up by the ependymal cells covering the walls of the ventricles and the surfaces of the choroid plexus. Not all, but many of the ependymal cells contain the pigment particles in varying number. The minute and larger grains occur also within cells in the subependymal layer, in close proximity to the ventricles. The latter pigment-containing cells do not seem to have emigrated from the interior of the ventricles, so it is considered probable that the pigment has passed from the ependymal to the subependymal cells. A small quantity of pigment occurs also in the superficial perivascular spaces in the cortex, but not in the spinal cord. The pigment penetrates to the interior of the ganglia with difficulty, along two courses: first the pia capsular investment, second the septa of the nerve roots. About the pigment there is a marked cellular reaction, and pigment-containing leucocytes come to lie against or near nerve cells, but no wide diffusion occurs within the ganglia. The meninges of the brain and spinal cord show a rich cellular exudation containing pigment.

When an aseptic inflammation has been set up previously, and the animal etherized twenty-four hours after the pigment has been injected, the distribution is identical with that described. The single difference noted is a greater amount of pigment within the ependymal and subependymal cells of the ventricles. In the case of an animal in which the pigment was injected during the early paralytic stage of poliomyelitis and which was etherized twenty-four hours later, the cellular accumulations were greater because of the addition of the polynuclear cells to the usual mononuclear infiltration. The choroid plexus of the lateral ventricle showed marked lymphoid, nodular aggregations beneath the ependymal cells, and a rich leucocytic emigration outside. The quantity of pigment taken up by the ependymal and subependymal cells is somewhat greater than that observed in the other instances.

The experiments with the carmin may be interpreted as indicating that the ependymal cells in a living state can be entered by particulate substances. Whether the strictly normal ependymal cells take up and pass on, in the manner described, pigment particles cannot be deduced from the experiments, as the carmin itself causes an inflammatory reaction and consequently may act injuriously upon the cells. Aside from the presence of the pigment, the ependymal cells exhibit no morphological alteration. Apparently a previous inflammation, such as that caused by horse serum and the virus of poliomyelitis, has the effect of rendering the ependymal cells more permeable for the pigment.

PATHOGENESIS OF POLIOMYELITIS.

A consideration of the experiments described in this paper should deal with the question of the pathogenesis of poliomyelitis which they are believed to elucidate.

We are confronted with the problem as to the site of entrance of the virus of poliomyelitis into the human body, as well as the manner in which the specific lesions of the disease are produced. The latter question has already been cleared up in large measure.[9]

[9] Flexner, *Jour. Am. Med. Assn.*, 1910, lv, 1105. Flexner and Lewis, *Jour. Exper. Med.*, 1910, xii, 227.

It is now sufficiently obvious that the virus possesses affinity for nervous tissues in general, but for no element of these tissues in particular. The constancy with which meninges, blood vessels, interstitial parts, and nerve cells are affected indicates that they all react to the presence of the virus. On the basis of actual observations it cannot be stated that virus is attracted by the nerve cells, either alone or necessarily in advance of the other structures mentioned; while the experiments here recorded show that it is only when the virus is brought to the nervous organs otherwise than by the general blood that the tissues composing them are able readily to remove and attach it to themselves.

This latter fact is a cardinal point, and one from which we may derive valuable information on the pathogenesis and mode of infection of the disease.

It may be regarded as established that all intraneural means of infection are successful, and that the virus travels with more or less ease and certainty along the nerves to the interstices of the central nervous organs, probably utilizing the lymphatic channels of communication. The experiments given in this paper show that the central nervous organs, excepting the intervertebral ganglia, are incapable of removing the virus from the general blood prior to changes induced in the blood vessels and in the choroid plexus. They indicate, also, that in the monkey these preliminary lesions are of a nature that permits of differentiation from the lesions caused by the intraneural modes of infection. The lesions in human cases of poliomyelitis would seem to correspond with those caused by intraneural and not by intravenous inoculation.

In general it should be stated that the intraneural modes of inoculation are effective in proportion to the degree with which they bring the virus into intimate relation with the central nervous tissues. On that account intracerebral inoculation is the most effective, because it not only insures contact between the virus and the mechanically injured tissues, but also because it isolates the virus in the brain tissue, under conditions favorable to multiplication and gradual diffusion into the ventricles and cerebrospinal fluid. Intraspinous injection is somewhat less effective for the reason that a part, and sometimes perhaps all, of the virus may be carried into the gen-

eral blood before it can reach and become attached to the nervous tissues. Intranasal infection is, in keeping with the general statement made above, more certain in its results than subcutaneous or intrasciatic inoculation, because of the proximity of the short olfactory nerve fiber to the brain tissue. It may fail, because the virus is washed away before it reaches the olfactory fibers and can be carried to the brain. Possibly intraocular inoculation may prove among the most successful, because the virus cannot escape and has only a short distance to travel to the brain; while the vitreous humor may even prove a favorable medium for its multiplication.

In the main, under natural conditions, it is the upper respiratory mucous membrane that would most often become contaminated with the virus, and most readily favor its conveyance to the brain. This series of events is determined by the manner in which the virus is thrown off by the infected body,[10] by the fact of its presence upon the nasal mucosa, even in healthy persons in contact with cases of poliomyelitis,[11] and by the demonstration that it passes, on the whole easily, along the olfactory nerve fibers to the brain, medulla, and spinal cord.[12] Although the virus is conveyed to the nervous organs from without by the lymph, the distribution throughout the nervous system is, in large part at least, effected through the medium of the cerebrospinal fluid. Even when the virus passes from the blood into the nervous organs, it takes the indirect course through the cerebrospinal fluid. This important fact has been established, not only by the finding of the virus by inoculation tests in the cerebrospinal fluid[13] after a blood injection, but also through the prevention of infection by the injection of immune serum into the subarachnoid spaces after lumbar puncture following the intravenous infusion of the virus under conditions insuring infection.

[10] Flexner, *Jour. Am. Med. Assn.*, 1910, lv, 1105. Flexner and Lewis, *Jour. Exper. Med., loc. cit.*

[11] Flexner, S., Clark, P. F., and Fraser, F. R., *Jour. Am. Med. Assn.*, 1913, lx, 201.

[12] Flexner and Lewis, *Jour. Am. Med. Assn.*, 1910, liv, 1140. Flexner and Clark, *Proc. Soc. Exper. Biol. and Med.*, 1912–13, x, 1. Flexner, *Lancet*, 1912, ii, 1271; *Science*, 1912, xxxvi, 685. Landsteiner and Levaditi, *Ann. de l'Inst. Pasteur*, 1910, xxiv, 833.

[13] Flexner and Amoss, *loc. cit.*

Thus the experimental evidence, which is upheld by observations in human cases of poliomyelitis, supports the view that epidemic poliomyelitis is caused by the entrance into the body of its specific microbic cause or virus, through the upper respiratory mucous membrane to the olfactory lobes of the brain, from which by means of the cerebrospinal fluid it is distributed throughout the substance of the nervous organs; but, since the virus may reach the brain by way of any nervous channel, and even, although with great difficulty, from the blood, it is, of course, possible that in exceptional instances other modes of infection may arise.

SUMMARY.

The virus of poliomyelitis is capable of penetrating the retina without producing apparent injury, to reach the central nervous organs.

The virus injected into the blood is deposited promptly in the spleen and bone marrow, but not in the kidneys, spinal cord, or brain.

Notwithstanding the affinity which the nervous tissues possess for the virus, it is not removed from the blood by the spinal cord and brain until the choroid plexus and blood vessels have suffered injury.

The intervertebral ganglia remove the virus from the blood earlier than do the spinal cord and brain.

An aseptic inflammation produced by an intraspinous injection of horse serum facilitates and insures the passage of the virus to the central nervous organs, and the production of paralysis. The unaided virus, even when present in large amounts, passes inconstantly from the blood to the substance of the spinal cord and brain.

When the virus within the blood fails to gain access to the central nervous organs, and to set up paralysis, it is destroyed by the body, in course of which destruction it undergoes, as a result of the action of the spleen and, perhaps, other organs, diminution of virulence.

The histological lesions that follow the intravenous injections of the virus in some but not in all cases differ from those which result from intraneural modes of infection.

In escaping from the blood into the spinal cord and brain, the

virus causes a lymphatic invasion of the choroid plexus and widespread perivascular infiltration, and from the latter cellular invasions enter the nervous tissues. A similar lymphoid infiltration of the choroid plexus may arise also from an intracerebral injection of the virus.

The histological lesions present in the central nervous organs in human cases of poliomyelitis correspond to those that arise from the intraneural method of infection in the monkey.

The virus in transit from the blood through the cerebrospinal fluid to the substance of the spinal cord and brain is capable of being neutralized by intraspinous injection of immune serum, whereby the production of paralysis is averted.

Carmin in a sterile and finely divided state introduced into the meninges and ventricles sets up an aseptic inflammation, but is quickly taken up by cells, including ependymal cells. When an aseptic inflammation has been previously established by means of horse serum, or when the nervous tissues are already injured by the poliomyelitic virus, the pigment appears to enter the ependymal cells more freely.

The experiments described support the view that infection in epidemic poliomyelitis in man is local and neural, and by way of the lymphatics, and not general and by way of the blood. Hence they uphold the belief that the *infection atrium* is the upper respiratory mucous membrane.

EXPERIMENTAL TUBERCULOSIS OF THE CORNEA.*[1]

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When an attempt is made to study the tissue reactions against the tubercle bacillus in either the normal or the immune animal, difficulties of a technical character are at once encountered. The final picture in an animal infected with tuberculosis is extremely complicated, and it is impossible to resolve it into its functional elements. Methods of examination during life are practically limited to such general conditions as changes in activity, body-weight, and temperature. The great help derived in the study of other infections from the investigation of changes in either serum or cells of the blood does not touch the problem.

In the belief that it would be of great advantage to be able to study a discrete tuberculous lesion which could at the same time be kept under constant observation without the necessity of resorting, in each experiment, to the tedious technical procedures involved in microscopic examination, we have undertaken a renewed study of experimental corneal tuberculosis.

The work is still in progress. This paper is intended to present primarily the technical phases of the subject, and to indicate briefly the limitations and possibilities of this line of experimentation. Secondarily, there will be presented some facts to show that the corneal lesion is susceptible of influence in an appreciable degree by both local and systemic measures and conditions.

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[1] The experiments here published were used as the basis of a paper read before the Lænnec Society of Johns Hopkins University in April, 1912. Extraneous circumstances have interfered with the prompt publication of the matter. In the meantime the experiments have for the most part been repeated on a large scale in connection with chemotherapeutic studies. The results of the later experiments conform in all essential respects to those here described

The general features of the lesion of the cornea following inoculation with tubercle bacilli or tuberculous matter were described by Cohnheim and Salomonsen (1) in 1877. Their inoculations were made into the anterior chamber, and consequently their lesion did not develop in detail according to our description.

Other observers have used the corneal experiment to study microscopically the early development of the tubercle, but this is a matter with which we are not here immediately concerned.

Hansell (2) in 1879 inoculated the cornea, conjunctiva, and anterior chamber with tuberculous matter. He described the corneal lesion in its essentials with accuracy, both in its gross and microscopic features.

Krusius (3) has presented interesting data on experimental tuberculosis of the various parts of the eye. He has developed the fact that the cornea is less susceptible to infection than the anterior chamber and the vitreous humor. It is more susceptible than the lens. Krusius states that the incubation period of the lesion in the cornea is inversely proportional to the dose of culture used. This author notes that in order to get consistent results it is important to use a culture of known virulence and rabbits of equal resistance (same weight, age, etc.). In a general way our results agree with those of Krusius entirely, although we have developed them and have considered the subject from an entirely different point of view. In one or two points there is an apparent contradiction between our results and his. He states that generalization does not occur from the eye lesion even when a culture of bovine type is used. He also found that the lesions showed frequently a distinct tendency to heal. Our animals when they have been kept for a sufficient length of time have always shown tubercles in the internal organs. In but few instances have the corneal lesions shown any definite tendency to heal. These differences indicate that we have used cultures of higher virulence than those employed by Krusius.

The observations on the use of iodin locally have been indicated by the clinical use of this substance more or less regularly since the time of Lugol for the purpose of influencing tuberculosis in one or another of its manifestations.

In using calcium lactate we have followed the observations of Chiari and Januschke (4) on the inhibitory influence of soluble calcium salts administered generally on simple inflammations.

Our observations on benzol have been made under the influence of the experimental work of Selling (5), in which the pronounced leucotoxic action of this substance was developed.

TECHNIQUE.

In all the experiments reported, we have used rabbits. The animals have varied in weight from 1,500 to 3,000 grams, have been of various colors, and purchased from various sources. They were of no especial breed. For most of the experiments we have used a culture of bovine type, isolated from cervical glands in 1907. The culture retains a high degree of virulence, as 0.01 of a milligram intravenously injected will kill a rabbit of 2,000 grams in from

twenty to thirty days, with generalized tuberculosis. The culture was grown on glycerin agar, and the growth was used within fourteen days of the time of transfer. The culture was suspended in a salt solution to make a suspension as homogeneous as possible, and this was centrifuged at high speed for a short time to remove the macroscopic clumps. The suspensions varied in weight, but were, as a rule, made by suspending one glycerin agar tube in ten cubic centimeters of salt solution. The completed preparation was usually of sufficient density to be just completely opaque when contained in the ordinary twenty millimeter bacteriological test-tube.

The eyes before inoculation have been carefully anesthetized with cocain. When this is done, the injection can be made without restraining the animal in any way, and it gives no pain or discomfort. In the beginning we tried to fix the eye with a pair of forceps. We found later that the injection could be made equally well without it. The injury done by the fixation forceps frequently gave rise to secondary foci of infection which should be avoided. We have used a small record syringe, but we have found it better to use special needles that we have had made for the purpose. The most satisfactory are of iridoplatinum, of about gauge 26, and ground to a very short bevel, with a sharp point. The advantage of the platinum needle is that it retains a smooth surface free from rust, even after being sharpened repeatedly. This seems to be very important in making a successful injection. The ordinary steel needles can be used with success when they are new, but they cannot be used after sharpening or repeated boiling.

The injection is made by placing the point of the needle against the cornea in the position desired, holding the syringe in a plane parallel to the cornea, considered as a flat surface. The needle is then inserted by a succession of short, jerky pushes, in which only slightly more force is used than that exerted by allowing the weight of the syringe to bear on the needle point. By this means, the eye is rotated, and between each push is allowed to come back almost to its normal position. When the aperture of the needle is entirely covered by the corneal epithelium, pressure is put on the piston, and the suspension forced into the cornea. Considerable pressure is required. The fluid raises a small vesicle which is at once opaque.

We have not found it practicable to measure the amount of fluid injected, but a fairly uniform series of inoculations can be secured by watching the size of the opacity produced. We have had most uniform results when this measured about three or four millimeters in diameter.

It cannot be said that this technique is perfect. Occasionally, instead of raising a definite circumscribed vesicle, the fluid flows into the cornea along radiating lines, spreading widely from the point of puncture. In a certain number of instances the needle penetrated the anterior chamber. This accident, however, has grown much less frequent as we have gained experience. Moreover, it seems probable that the ultimate result varies, to a certain extent, according to whether the injection mass remains localized close beneath the corneal epithelium, or flows more deeply into the tissue. When the larger amounts suggested are injected, there seems to be less variation due to this factor. In certain instances, instead of injecting the material we have made the inoculation simply by pricking the cornea with the needle wet with the culture. The results of this procedure will be explained later.

In our examinations made subsequent to inoculation we have confined ourselves to the use of oblique illumination. We have found it advantageous to use artificial light because of the greater constancy, and have adopted a 40-watt Tungsten lamp with a ground glass bulb held about sixteen inches distant from the eye. With this we have used a small hand lens.[2]

We have, of course, made careful notes of the development of the lesion, but have found that frequently we save much time and secure, on the whole, more useful records by charting the conditions from day to day.

[2] Dr. Thomas B. Holloway has recently been over the methods of examination of the eye with us and has pointed out the possible usefulness of the corneal loop and the ophthalmoscope. We have not used these instruments extensively, so far as the results at present recorded are concerned, but they will probably add to the accuracy of our examinations in the future. It is likely that the use of these more accurate methods of examination would modify our statements only as they concern the time of appearance of the earliest vascularization of the cornea.

DEVELOPMENT OF THE CORNEAL LESION.

The Effect of Large Doses of Tubercle Bacilli.—When a heavy suspension of tubercle bacilli is injected into the cornea in such a way as to make a distinctly circumscribed infiltrated area of three to four millimeters in diameter, the first result, as has been said, is a circumscribed opacity, which probably marks fairly well the limits of the infiltration. At the end of twenty-four hours this opacity has usually almost disappeared. At this period one is able by careful examination to see the defect in the cornea that marks the point where the needle penetrated, and extending from this defect a very short way into the cornea is a very fine, whitish or brownish white line. At this period, the sclera and bulbar conjunctiva will have their normal appearance, and there will be no evidence of any irritation of the eye. In from three to seven days, depending on various factors, there is a noticeable change. A small increasing opacity of grayish white color develops at the point of inoculation on the cornea, and the conjunctiva of the bulb becomes increasingly injected from day to day. On the seventh day, for the average, the opacity at the point of inoculation has a diameter of about one millimeter. The relation of the size and rapidity of growth of the opacity to the appearance and development of the injection of the bulbar conjunctiva is not perfectly regular, although it is fairly so. As a rule, the conjunctival congestion begins at about the time that the first visible increase in the exudate at the inoculation point shows itself. After several more days, the conjunctival congestion having in the meantime become very intense, loops of newly formed capillaries appear in the cornea, pushing out from the margin towards the inoculation point, and the exudate at the inoculation point continues to increase. The loops of vessels extend further and further into the cornea, until finally they come into contact with the exudate. The congestion of the bulbar conjunctiva becomes complicated by congestion of the conjunctiva of the lids. The lids swell and there is more or less of an increased secretion, which is at times white, at times yellowish. It is usually sufficiently fluid to remove itself spontaneously, but occasionally it becomes sticky and glues the lids together. This does not often occur

before the third week after inoculation. At about the time that the corneal vascularization or pannus comes into contact with the exudate at the point of inoculation, the corneal epithelium over the exudate usually breaks through leaving an open ulcer.

The formation of the pannus takes place in a regular way. The first vessels formed are capillaries and an advancing margin is maintained as a capillary zone. Behind the advancing margin there is a tendency for the pannus to thicken to a definitely raised rather pale zone of granulation tissue. Between this area and the corneoscleral junction the vessels are gradually reduced in number and increased in size until there are formed several main trunks of supply to the more central lesion. These supply trunks run through a cornea which has become partially clear again. In the later stages, with the advance of the exudate and the invasion of the pannus by it, the peripheral clear zone again becomes obscured and filled with granulation tissue.

When large doses are used, the pannus never tends to invade, in any marked degree, the mass of exudation. On the contrary, the exudate gradually extends and infiltrates the pannus. Most of our experiments have terminated at this point. The exceptional instances which have been kept longer under observation will be commented on in later paragraphs in connection with special points. With the large dose in the untreated eye, the vascularization of the cornea usually begins between the eighth and twelfth days.

The Effect of Small Doses of Tubercle Bacilli.—With the results following large doses, we have contrasted the effects of a minimal inoculation. They have been obtained, as before stated, by simply wetting the needle with the culture and pricking the cornea. In making the inoculation in this way it is essential that the epithelium be pierced. If the epithelium be merely superficially scratched, the inoculation is not effective. With the small dose, there come into play the same factors that have been described for the large dose. Very essential differences, however, are to be noted. The rate of progress of the lesion is much less uniform. The ultimate appearance of the lesion, in the majority of cases, is much the same, but in details of the first importance there are variations. It is especially by the use of this method of inoculation that one can deter-

mine differences in individual resistance of the animal. In old rabbits the changes occur much more slowly than in young rabbits. It has occasionally happened, in using this method on old rabbits, that the pannus has completely infiltrated the mass of exudation at the point of inoculation. In the young rabbit it has happened conversely that the pannus has not only been invaded by the mass of exudation, but that it has actually been involved extensively in the ulcer, and has been almost completely eaten away. Various intermediate conditions are encountered.

INFLUENCE OF VIRULENCE OF CULTURE.

We have not so far studied extensively the action of different cultures on the cornea. However, some inoculations were made with cultures of human type which are, of course, non-virulent for rabbits. The large dose before described produced the same end result that the large dose of the virulent culture did. Smaller doses or doses of less virulent human culture produce a lesion which is smaller and which shows a great tendency to heal spontaneously. The lesion in its development follows the general course of that described for the bovine type culture with some differences which serve to place it between the bovine lesion and that about to be described for the non-virulent acid-fast bacilli. The immediate reaction to the human type is somewhat more rapid as a rule. The blood vessels begin to form more quickly but the progress of the lesion is less rapid. While the virulent culture generalizes from the eye as a focus, the human type of culture does not generalize. We hope later to develop this phase of the subject.

CONSEQUENCES OF PERFORATION.

If the cornea is perforated at the time of inoculation or, as has often happened in our series, the ulceration of the cornea occurs internally as well as externally, an infection of the anterior chamber results. The consequences of such infection have been sufficiently described by others. We wish to note, however, one or two points which are of interest by contrast with the pure corneal infection, and which chiefly concern the pannus. The typical pannus, which

is formed as a result of the infection of the cornea, begins at a single point on the corneoscleral junction, extending centrally and broadening out from this point. If the injection is so placed that it is somewhat above central, the pannus always begins at the upper margin of the cornea. When the lesion has become quite extensive, a second pannus may appear starting from below. Placing the injection forward or back of central throws the point of beginning of the pannus either somewhat forward or back, as the case may be. It is an interesting point that when the injection is either precisely central or slightly below central, the first pannus will still form above. From the moment that the cornea perforates internally, however, a circular pannus begins to form. We have controlled this point by injecting the anterior chamber in such a way that the cornea was not injured; that is, by passing the needle through the sclera from behind. Here the pannus is also circular, and no difference between the upper and lower margins can be noted. These differences presumably depend on the circulatory channels in the cornea, but we have at present no more precise explanation for them.

THE EFFECT OF KILLED CULTURES OF THE TUBERCLE BACILLUS.

When we had proceeded with this work for some way, we were led to consider how much of the effect produced by the inoculation was specific for the tubercle bacillus, and how much might depend on the non-specific factors. Before trying anything more remote, we used the same culture killed by heating for one half hour to 60° C. When such a killed culture is injected in about the quantity of a large dose of living culture, the first effect is the same. At the end of twenty-four hours a difference appears, in that the bulbar conjunctiva is usually pronouncedly injected, and the eye appears to be definitely more irritated than by the injection of the living culture. The inoculation point becomes marked out by an exudate in about the same way as with the living culture, but this exudate seldom reaches more than pinhead size. On the fourth or fifth day, as contrasted with the eighth to the twelfth day for the living culture, loops of vessels appear in the cornea. They appear

at the situation favored by the vessels appearing in response to the living culture. They extend very rapidly into the cornea as a thin leash which on the sixth or the seventh day reaches nearly to the point of inoculation. The base of the leash never broadens; it is usually about three to five millimeters in width. From the seventh day on these vessels seem rapidly to disappear. By the tenth day nothing more of them can be seen in the cornea by our methods of examination. No permanent pannus is formed. The exudate at the inoculation site may be entirely reabsorbed, or may persist for months as a pinhead-sized scar. The vessels do not, however, really disappear, at least not within a period of two months. They cease to functionate. This can be shown by making a fresh inoculation with either dead or living cultures near the old scar. Under these circumstances at the time when the congestion of the sclera becomes well marked, the old corneal vessels suddenly fill and the new formation of vessels in consequence of the second inoculation takes place from the end of the old leash.

For obvious reasons we have compared the lesions so far described with the corneal lesion produced by other bacteria. The strains of *Staphylococcus aureus* and *Bacillus typhosus* used, when inoculated on the cornea near its center, produce a rapid local exudation. There is, however, no tendency to vascularize the cornea and the exudate finally resolves more or less completely leaving a relatively small opacity at the inoculation site.

The acid-fast bacteria other than *Bacillus tuberculosis* give a lesion which in severity and time relation develops much more like the lesion caused by the dead tubercle bacilli than by the living. The exudation is small, the vascularization commences early, and the vessels cease to functionate after a short time. The lesion usually resolves, leaving only a small scar. We have used in this connection the bacillus of timothy, the butter bacillus of Grassberger, Korn's grass bacillus D., the bacilli of frog and fish tuberculosis, an old isolation of the bacillus of avian tuberculosis, and the chromogenic *Bacillus lepræ* of Duval. The last of these cultures has sometimes shown a more progressive and persistent lesion than the others. The difference between *Staphylococcus aureus* and *Bacillus typhosus* on the one hand, and *Bacillus tuberculosis* and the acid-

fast bacteria on the other, in capacity to stimulate the formation of vascular tissue in the cornea is striking. It is, however, a purely quantitative difference or one which is not manifest under more general conditions. This is shown when *Bacillus typhosus* or *Staphylococcus aureus* are inoculated, avoiding the cornea, into the anterior chamber through the sclera. As a consequence of the anterior chamber infection a vascular, circular pannus quickly forms on the cornea.

As described, the corneal lesion due to the inoculation with the tubercle bacillus proceeds in an orderly fashion through certain clearly defined stages and presents in some degree an opportunity for an analysis of the processes involved. Certain experiments were now undertaken to determine whether or not the course of the lesion could be influenced by the local and general administration of various substances. As we hope to develop this phase of the subject in greater detail in the future we shall now merely present our results in outline in so far as they show that the lesion can be modified.

Iodin, Locally Applied as Lugol's Solution.—If the conjunctival sac be washed daily with Lugol's solution the early development of the lesion is in no way influenced. In many instances the pannus when well formed thickens to an unusual degree. The secondary thinning out of the vascular tissue near the corneoscleral margin does not take place. In order to attain this effect the concentration of the iodin in the solution and the duration of the exposure must be sufficient to produce a distinct inflammation when applied to the healthy control eye. The effect may be in large measure an additive one. We have never seen the pannus invade the central exudate under the influence of iodin.

Tuberculin.—Koch's old tuberculin given intravenously in large or small doses or instilled into the eye is without effect on the early development of the lesion. We have not tried its effect later when panophthalmia exists, and our results in no way contradict those of previous observers who have made out a favorable influence from the treatment of experimental and clinical tuberculosis of the eye with this preparation.

Calcium Lactate.—Calcium lactate administered intravenously or subcutaneously in doses sufficient to intoxicate the animal severely is able to inhibit the corneal process in considerable degree. The central exudation perhaps increases more slowly than usual, although this is not perfectly clear. The vascularization of the cornea is somewhat delayed in point of time and proceeds more slowly and much less vigorously than usual.

Benzol.—The subcutaneous administration of benzol in olive oil to the point of severe intoxication decreases the amount of exudation to the inoculation point and causes the vascularization of the cornea to develop slowly and feebly. This experiment like that with calcium lactate cannot be carried to a satisfactory conclusion because it is necessary to give the substance in doses certainly although slowly fatal in order to get demonstrable results. The control blood counts have shown that only in those cases in which the leucocytes were reduced very low has there been an appreciable influence on the development of the lesion.

Intercurrent Disease.—In several instances we have inoculated recently purchased, supposedly normal animals which in the first few days developed an acute illness followed by a period of extreme emaciation. In these animals the development of the corneal lesion was also slow and without vigor. The exudation at the inoculation point was inhibited and until this was well developed there was no vascularization of the cornea.

DISCUSSION.

The foregoing experiments taken in a general sense can hardly be said to differ radically from certain of those previously performed by others. Moreover, when we consider them in detail and attempt to formulate their meaning it is often difficult to be sure that what we have learned is not already fairly well understood by those closely familiar with the pathology of tuberculosis. In spite of a degree of uncertainty as to the final value of such considerations, it is our purpose to state in our own language the point of view at which we have arrived by way of this work and to indicate some of the problems which are defined by it.

Tuberculosis is classed by systematic pathologists as an infectious

granuloma. The justice of this classification is emphasized by these experiments. The corneal lesion consists of a mass of granulation tissue the various elements of which are present in variable proportions at different stages in the development of the disease. The experiments recorded in which the action of *Bacillus typhosus* or *Staphylococcus aureus* is contrasted with that of the tubercle bacillus on the cornea indicate that the latter much more readily gives rise to the formation of blood vessels. This power of stimulating blood vessel formation is also possessed by other acid-fast bacilli. As shown by the results of anterior chamber inoculation, however, the difference is one of quantity rather than of quality. *Bacillus typhosus* and the pyogenic cocci under proper conditions may likewise stimulate blood vessel formation. As a large exudative lesion produced on the cornea with *Bacillus typhosus* may fail to give rise to the new formation of capillaries, while even a minimal lesion with dead tubercle bacilli or with non-virulent acid-fast organisms of other kinds leads to their rapid formation, we are probably justified in believing that the difference is a large one and perhaps of vital importance in the development of the disease. The corneal lesion develops in an orderly way. The irritation due to the injection is recovered from, is followed by a latent or incubation period, and this in turn is followed by the development of a local exudation. Not until the exudate has become definite does the formation of blood vessels begin. In experiments such as those with benzol and calcium lactate in which the primary exudation is limited or suppressed, the blood vessels fail to develop or develop very feebly. Presumably if it were possible to prevent the exudation entirely, the vessels would never develop. Expressed in terms of the prevention of the disease, these results are of little significance. While it is possible to say in these terms that the development of a tuberculous lesion has been definitely delayed by these agencies, yet it has been by methods that are so severe in their general effects that even the experiments can hardly be continued to a satisfactory conclusion.

From another point of view, however, the matter has interest. In the well developed corneal lesion the tubercle bacillus is present in enormous numbers. Several of these inhibitive experiments have lasted from two to three weeks, and in this time the cultures we

have used on culture medium attain a large growth. If in this time there was a great growth of the organism in the transparent cornea it might be expected to become visible; or if the bacteria did not become visible as a colony, the cornea should develop a local defect. As a matter of fact no visible change occurs in the absence of exudation and it seems to be a fair conclusion that we are dealing with a true condition of latency, one in which the bacillus grows little if at all, and in which the tissues are practically unchanged. Stated in another way, the conclusion is indicated that there is nothing in the fixed tissues of the cornea to provide for food for the bacillus and little or nothing about the living tubercle bacillus which does injury to these tissues. With the coming of the cellular exudate the case is entirely different. The products of cellular degeneration furnish food for the bacillus and the fixed tissues are attacked either by the products of the growth of the bacillus in this pabulum or by the products of cellular activity or cellular degeneration. From this heterogeneous combination also, substances are set free which give a stimulus for blood vessel formation and doubtless for the proliferation of other fixed tissue cells.

If this reasoning is in any measure correct, and we believe that it is largely so, we are forced to agree with those who have held that tuberculosis is primarily an exudative inflammation and secondarily proliferative in its character. More than that, we are led to the belief that the cellular exudate, protective though it may be in purpose, is defective and is destructive in its actual influence. Even the phagocytic activity of the cells of such an exudate may probably serve to distribute living bacilli and thus spread the disease to fresh areas.

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THE EXCRETION OF NITROGEN IN FEVER.*

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That there is no qualitative difference between the metabolism of fever and normal metabolism, and no ground for believing that proteins are broken down in infective fevers by the action of toxins upon the protoplasm of the cell, are the conclusions to which Krehl and Grafe (1) have come as the result of work recently done in the laboratories of the Heidelberg clinic.

The evidence adduced by Grafe (2) upon the meaning of the increased nitrogen excretion in fever is partly derived from measurements of the respiratory exchange in fever patients on a fluid diet yielding about 1,200 calories and containing about 5 to 6 gm. of nitrogen, and calculation, from the nitrogen output, of the ratio of the energy derived from protein oxidation to the total amount of energy liberated. This ratio he finds to be similar to that observed in starvation. Partly too (3), from the fact that, if a sufficiently liberal estimate of the energy needs of such patients be made, about 50 calories per kilo, and this amount be supplied in the food, mostly in the form of carbohydrate, nitrogenous equilibrium may be maintained in fever, in most cases when less than 1.4 gm. of protein per kilo of body-weight are given. This amounts, he argues, to proof that the loss of nitrogen often observed in fever is due to the increased evolution of energy in fever and the diminished intake of food. He claims support for this view also from the results obtained by Shaffer and Coleman (4), who prevented loss of nitrogen in typhoid fever by administering very large quantities of carbohydrate. More recently Pfannmüller (5) has succeeded in reducing the output of nitrogen in fever as much as 40 per cent. by adding to an otherwise constant diet 500 gm. of sugar, whereas in patients on this diet whose temperature was normal the addition of this amount of sugar reduced the nitrogen output only about 10 per cent., showing that the largest part of the loss of nitrogen in fever is due to the deficiency of carbohydrate.

It seems clearly established at any rate from all these results that when a liberal supply of carbohydrate can be given it may reduce the destruction of tissue protein in fever. But this result does not necessarily imply that the destruction of protein that would otherwise take place is due merely to a deficiency of calories in the food;

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the presence of large quantities of carbohydrate may affect the course of chemical processes in the body in other ways than merely by furnishing material that is easily oxidized. It is not altogether in harmony with what we know that it should be necessary to supply 50 calories per kilo to a patient in bed even if he is in fever. There is a fair consensus of evidence that the respiratory exchange is increased in fever often 20 or possibly 30 per cent., seldom more than that, and also that 30 calories per kilo is a sufficient supply for a patient lying in bed; if 30 per cent. be added to meet the calls due to the fever, 40 calories per kilo should theoretically suffice in most cases of fever. In Shaffer and Coleman's experiments, it was necessary so to drench the patients with carbohydrate that the calories supplied amounted to 80 or even 100 per kilo.

In some ways the most valuable study of metabolism in fever that has yet been carried out is that of Likhatcheff and Avroroff (6). A woman suffering from malaria was examined in the calorimeter on days on which attacks occurred, and also when her temperature remained normal, but conditions were otherwise similar. With a normal temperature her heat production was 32 calories per kilo per day. On a day when her temperature rose about 3° C. to 39.3° heat production rose to a little more than 35 calories per kilo.

It is difficult to see why, in order to prevent so small an increased evolution of energy as this resulting in the destruction of proteins in the body, it may be necessary to supply energy at a rate two or even three times as great as this, most of it in the form of carbohydrate, if the protein is attacked merely because there is not carbohydrate available.[1] The experiments of Kocher (7) recently reported showed that a diet of 70 calories per kilo containing only 2 gm. of nitrogen, which in patients whose temperature was normal gave an output of nitrogen amounting only to 3 gm., resulted in the loss of much larger amounts of nitrogen, up to more than 20 gm., in the case of patients in fever.

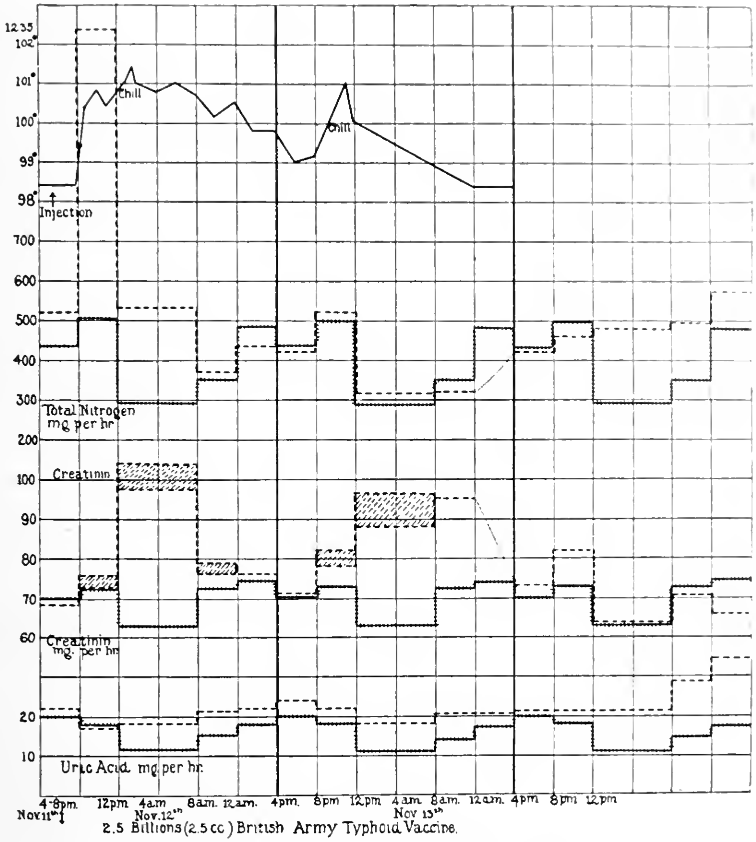
[1] It is true that this may not be a fair way of looking at the case in point. The energy output for the day on which the fever occurred amounted, when reckoned for the twenty-four hours, to only 10 per cent. more than on a normal day. But a valuable feature of the experiment is that the effect is followed not merely by the coarse method of twenty-four-hour periods, as is the case with most work on fever. A fine adjustment to two-hour periods was made use of, and it becomes apparent that between the hours of 3 and 6 A. M. the rate of heat production rose to 110 calories per hour when at these hours with a normal temperature it would have been only about 60 calories per hour. Such a sudden short burst of racing heat production might possibly be supposed to overstrain the immediately available supply of carbohydrate, and temporarily necessitate recourse to protein material; only it must be remembered that still greater departures from the usual rate of heat production, in muscular exertions, can be borne, as is well known, without any such result.

The principal difficulty in the study of metabolism in fever consists in the establishment of proper controls with which to compare the abnormal change. Controls obtained from the same individual during convalescence are not always necessarily satisfactory, and they are generally the best that can be obtained. The ideal condition would be to have the subject under examination for some days before the onset of the fever and to be able to keep the diet constant all the time. In the case of malaria this might be possible, but hardly in that of any other disease. But something may be done with the febrile reaction induced by the injection of toxins. Two experiments of this kind done on the same subject with typhoid vaccine were reported by Leathes in 1907 (8), and we have done two more on two other subjects with typhoid vaccine and four on four different subjects with tuberculin following essentially the method employed by him; we have also examined a patient suffering from malaria.

The procedure adopted consists in establishing the normal rates at which total nitrogen, creatinin, and uric acid are excreted by the subject at different periods of the day, by collecting the urine at fixed hours several times daily for several days on a constant diet. After a week the average output for each period of the day is calculated and plotted on squared paper representing the normal rate of excretion for that individual on that diet. The injection is timed so as to bring on the reaction in the evening and the maximum temperature in the night so that the intake of food is not interfered with at all and the disturbance of nutrition that it is otherwise so hard to allow for is entirely eliminated. On the next day the temperature has generally fallen; no meals are missed and the diet remains the same. The rates at which total nitrogen, creatinin, and uric acid are excreted during each period following the injection are then plotted on the same paper with the normal average rates previously obtained, and any departure from the normal becomes at once apparent. In this way the changes effected can be followed more minutely, and pronounced departures from the normal course can be detected, which being of short duration might otherwise be lost sight of in averaging up the whole output for twenty-four hours.

The results obtained are presented in the form of curves in text-figures 1 to 8. Text-figure 3 gives for comparison the results of

one of the experiments referred to above and previously reported by Leathes. The abscissæ represent time, divided into periods corresponding to the times at which the samples of urine for analysis were collected, generally every four hours. The ordinates give



TEXT-FIG. 1.[2] Case 1. Typhoid vaccine.

the hourly rate of excretion of either total nitrogen, creatinin, or uric acid, as indicated on the scale at the left hand side. In each case the dotted interrupted line shows the hourly rates for each period

[2] In the text-figures, the black lines represent the normal rate of excretion in mg. per hour of uric acid, creatinin, and total nitrogen, being the average of several days under fixed conditions, divided in four-hour periods. The dotted lines represent the rate in mg. per hour following the injection.

following the injection; the average normal hourly rates calculated from six to eight days on the same diet preceding the injection are shown as continuous lines (in text-figure 1 these lines have cross markings), and being normal averages repeat themselves every twenty-four hours. The charts for convenience have black vertical lines running through them dividing them into periods of twenty-four hours. The temperature curve is given so as to show the relation of the changes in the rates of excretion to the changes in body temperature.

The diet was in most cases milk and bread and butter; in some cases it included eggs and cheese or fruit. No meat or meat broths were included in any case. In all cases as far as possible the composition and amount of food and liquid taken were the same on each day of any given experiment and they were taken at the same hours throughout that experiment.

Creatinin was estimated by Folin's colorimetric method with bichromate solution for standard; uric acid by his modification of Hopkins' method; total nitrogen by Kjeldahl's method. Creatin was looked for in all cases except the experiment charted in text-figure 3; when found the amount is indicated by a shaded area in the creatinin curve.

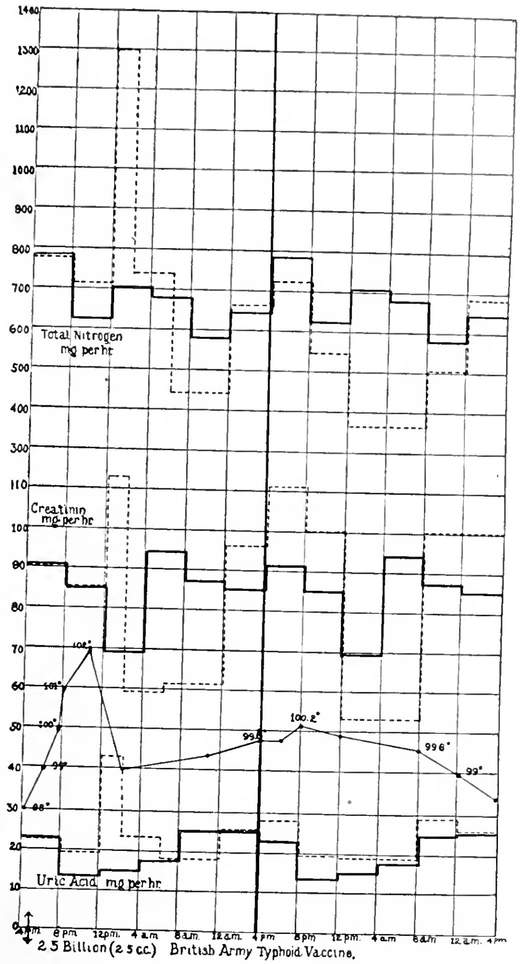
The time at which the injection was given is marked by an arrow on the chart.

If now the first three curves which give the results of experiments with typhoid vaccine are consulted, it will be observed that they agree in showing a sharp rise in the excretion rates synchronizing closely with the rise of temperature, and that they agree also in showing a greater change in the excretion of uric acid than of either creatinin or total nitrogen. When the reaction occurs the excretion of uric acid in case 1 is increased more than 60 per cent. for eight hours, and for more than twenty-four hours after that remains uniformly increased. In case 2 it is nearly trebled for two hours, and in case 3 it is more than trebled for eight hours and remains above the normal for several hours after that.

In table I the quantities excreted in twenty-four-hour periods after the injection, as compared with the normal twenty-four-hour average, are given. It illustrates how a marked reaction may be

missed if the whole of the urine for twenty-four hours is analyzed together, most notably in case 2.

In case 1 the uric acid in the blood was estimated on three days at the same hour by Folin's colorimetric method. On two days before



Text-Fig. 2. Case 2. Typhoid vaccine.

the injection the amount found in double estimations was 0.82 and 0.81 of a milligram per 100 cubic centimeters on the first, and 0.80 and 0.77 on the second. On the day when the injection was given,

TABLE I.

Typhoid Vaccine.

Total Excretion in Periods of Twenty-Four Hours.

	Total nitrogen.		Uric acid.		Creatinin.	
	Gm.	± per cent.	Gm.	± per cent.	Gm.	± per cent.
Case 1. N. C. S.						
Normal average	8.9		0.36		1.64[3]	
1st 24 hrs. after injection	14.4	+61	0.48	+33	2.02[4]	+23
2d 24 hrs. after injection	9.2	+3	0.48	+33	2.05[4]	+25
3d 24 hrs. after injection	11.8	+33	0.60	+67	1.68[3]	+2
Case 2. K. M. B. S.						
Normal average	16.1		0.46		2.10[3]	
1st 24 hrs. after injection	16.8	+4	0.55	+20	1.90[3]	−10
2d 24 hrs. after injection	13.0	−20	0.56	+21	2.10[3]	
Case 3. J. L.						
Normal average	7.6		0.44		2.07[5]	
1st 24 hrs. after injection	9.8	+28	0.71	+61	2.24[5]	+8

and seven and a half hours after it, the amount found was 1.75 milligrams per 100 cubic centimeters. In the urine that had been excreted during those hours the uric acid corresponded, as may be seen in text-figure 1, to the amount excreted at that time on normal days; but immediately after that the rate of excretion was increased and remained so for more than forty-eight hours. This result shows that the increased excretion cannot be accounted for merely by increased renal activity, but corresponds to a really increased production.

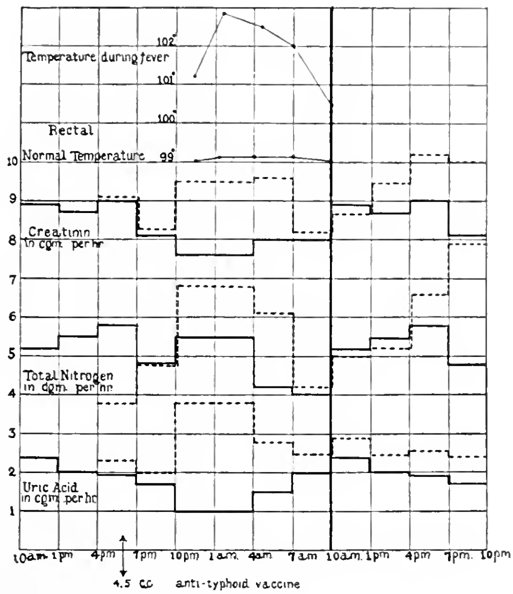
We find it difficult to reconcile entirely such results as these with the conclusions to which Krehl and Grafe have come with regard to the interpretation of the increased nitrogen excretion in fever. It is difficult to see how these marked reactions can be put down to a sudden deficiency of carbohydrate; the subjects were in good condition, well nourished, strong, healthy, and capable at any moment like other healthy subjects of doubling or trebling their ordinary rate of discharge of energy by muscular exertions without showing any such change in the nitrogen excretion. Moreover, it appears to us that there is evidence of a qualitative change in metabolism during

[3] No creatin found.

[4] Including creatin.

[5] Creatin not estimated.

a febrile reaction to be detected also in the way in which the uric acid is more affected than other forms of nitrogenous excreta. The reaction of the body to the typhoid toxin seems to consist not in a rise of temperature due, so far as it is due to increased heat production, to a general acceleration of metabolism qualitatively the same as that which was proceeding before the reaction was excited, but to chemical processes of a special kind. Whether these processes are merely certain special factors in normal metabolism that are necessarily exaggerated in the heightened activities, nervous or muscular

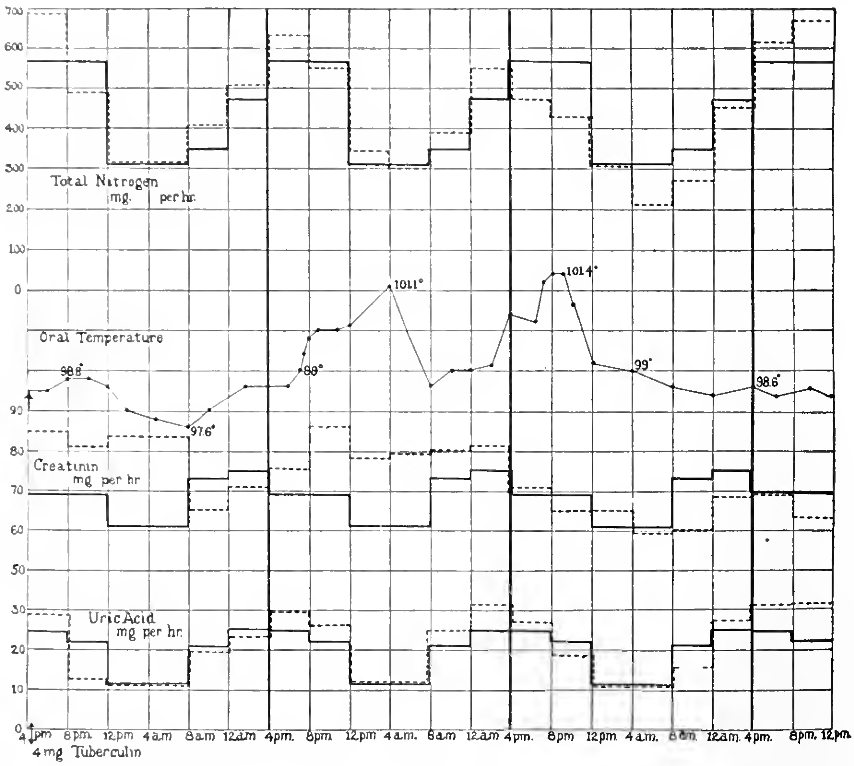


Text-Fig. 3. Case 3. British Army typhoid vaccine.

or of whatever nature they may be, that are involved in the reaction, or whether they are toxogenic and due to the damage done to cell protoplasm by the toxin, it is not possible from these data alone to decide. Possibly a comparison of the results of the injection of typhoid vaccine with the changes brought about by tuberculin, to which we now turn, may help to decide this question.

The results of tuberculin injections when studied in this way in four subjects, two of them normal, one slightly, the fourth

severely infected, are given in text-figures 4 to 7 and table II. It is clear that the reaction to this toxin is slower than the one already dealt with. Except in the case of the subject who was severely infected (case 7), about twenty-four hours elapse before the temperature rises, and the rise of temperature when it occurs tends to be less. The abrupt change in the rates at which nitrogenous ex-



TEXT-FIG. 4. Case 4. Tuberculin. Normal subject.

creta are turned out, that accompanies the prompt rise of temperature after typhoid vaccination, is missed in cases 4, 5, and 6, which reacted slowly, and is noticeable in case 7, which reacted more rapidly to tuberculin, only after the first injection. But in that instance the rise of temperature was little more than 1° F., and similar temperature reactions to a second and third dose, though these doses were larger, were accompanied by less definite changes in the excretion.

TABLE II.

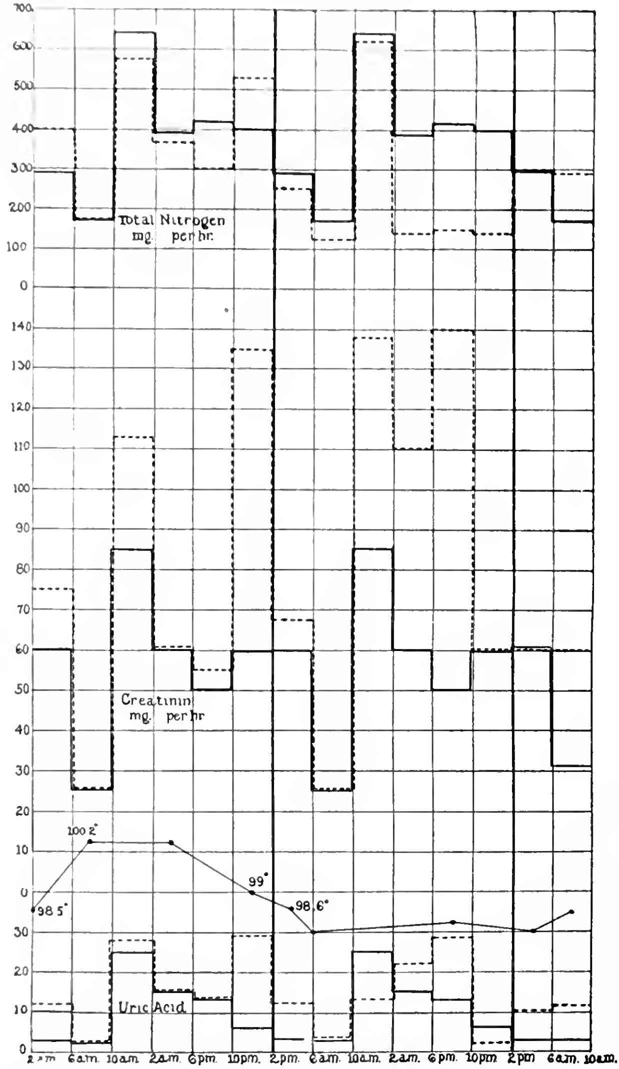
Tuberculin.

Total Excretion in Periods of Twenty-Four Hours.

	Total nitrogen.		Uric acid.		Creatinin.	
	Gm.	± per cent.	Gm.	± per cent.	Gm.	± per cent.
Case 4. N. C. S. (No tuberculosis)						
Normal average	10.2		0.45		1.6	
1st 24 hrs. after injection	10.4	+ 2	0.41		1.8	+13
2d 24 hrs. after injection	10.9	+ 7	0.52	+15	1.9	+19
3d 24 hrs. after injection	8.3	−19	0.41	− 9	1.4	−13
Case 5. C. L. (No tuberculosis)						
Normal average	8.9		0.26		1.3	
1st 24 hrs. after injection	12.6	+42	0.46	+77	1.9	+46
2d 24 hrs. after injection	8.8		0.26		1.8	+40
Case 6. W. P. (Slight tuberculosis)						
Normal average	9.8		0.22		0.84	
1st 24 hrs. after injection	10.5	+ 7	0.30	+36	1.10	+31
2d 24 hrs. after injection	9.9		0.34	+55	0.94	+12
3d 24 hrs. after injection	10.7	+ 9	0.24	+ 9	0.85	
Case 7. R. K. (Marked tuberculosis)						
Normal average	12.7		0.46		1.22	
1st 24 hrs. after injection	13.4	+ 5	0.50	+ 9	1.24	
2d 24 hrs. after injection	12.6		0.46		12.5	

The change in the output for twenty-four-hour periods shown in table II is considerable in cases 4, 5, and 6 in regard to creatinin, in cases 5 and 6, but less so in case 4 in regard to uric acid, in case 5 and less so in case 6, in regard to total nitrogen. Case 4 showing the reaction to tuberculin in the same subject as case 1 to typhoid vaccine is particularly instructive, especially as the temperature rose to the same level in both experiments, and the marked effect on the output of uric acid in the reaction of this subject to typhoid vaccine is missed in the reaction to tuberculin. The reaction to typhoid was accompanied by definite rigors, that to tuberculin was not. There does not therefore appear to be the same uniformity in the reaction of different subjects to tuberculin as in the reaction to typhoid vaccine. And the most striking feature in the reaction to typhoid vaccine, the sudden change in output of all the forms of nitrogenous excreta in-

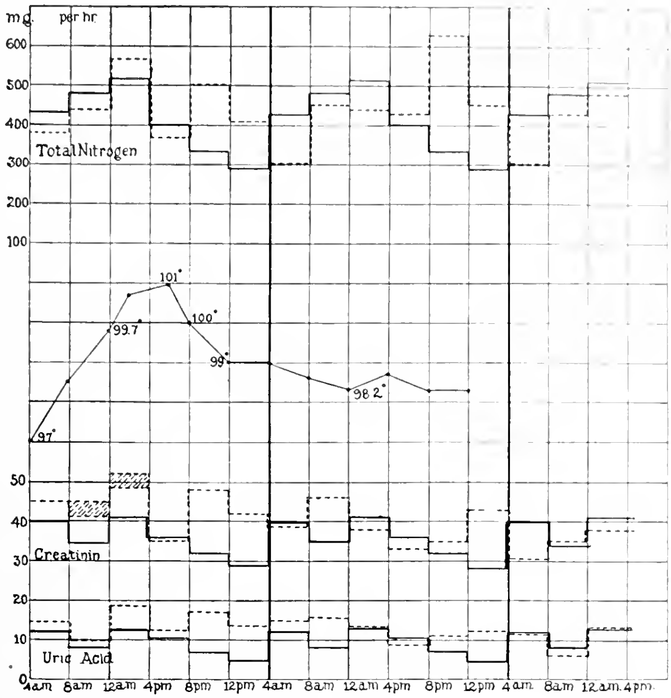
vestigated, accompanying the rapid rise of temperature, is not to be observed with the slower reaction to tuberculin. But in the reac-



TEXT-FIG. 5. Case 5. Tuberculin, 1 mg. eighteen hours before. Normal subject.

tion to tuberculin as in that to typhoid vaccine the changes that occur in the output of nitrogenous substances are such as to make it diffi-

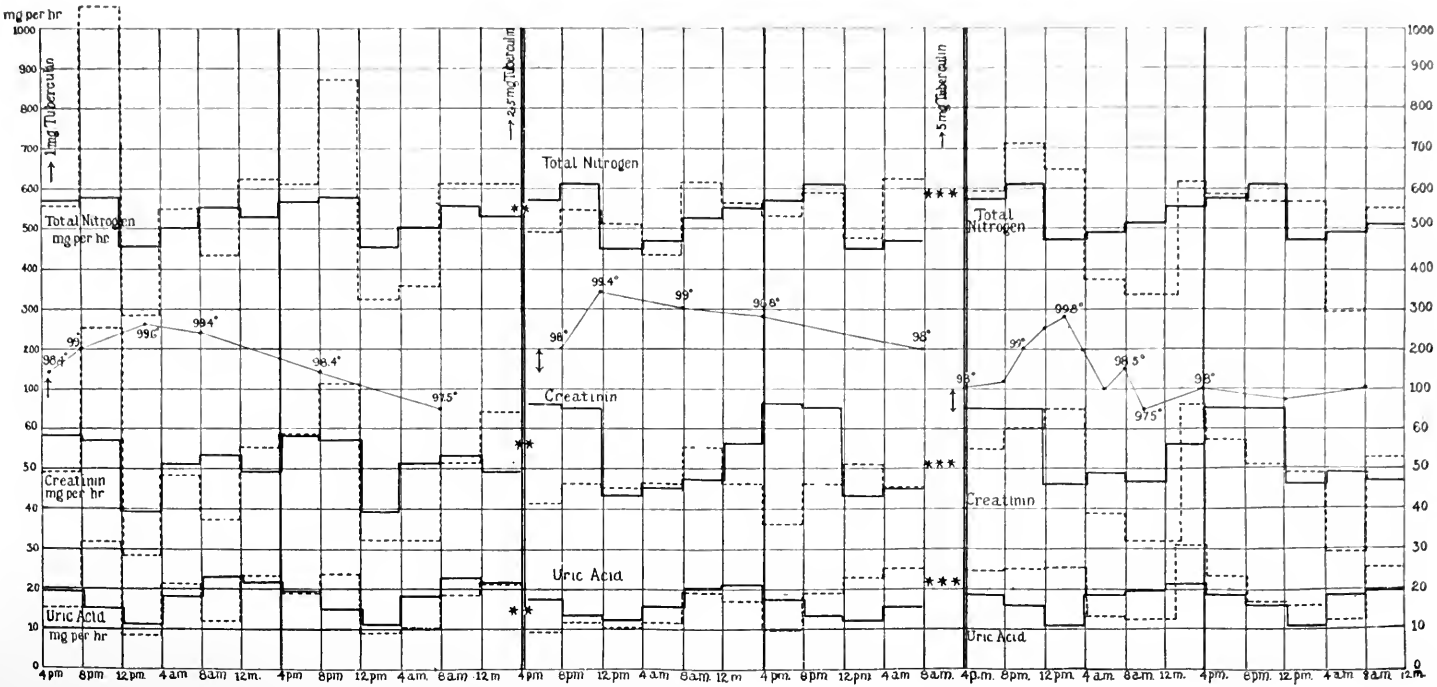
cult to concur in the conclusion that there is no qualitative difference between the metabolism in fever and normal metabolism. Whether the other fact which these experiments bring out, namely that the effects of the two toxins are different from each other in certain respects, should be interpreted as showing that the increased output is not due to increased activities involved in the process of raising



TEXT-FIG. 6. Case 6. Tuberculin, 2 mg. sixteen hours before. Subject suffering from slight tuberculosis.

the body temperature, and involved therefore alike whichever toxin was used, but is due rather to the two poisons attacking the cell protoplasm in ways that are different, though both of them cause a toxogenic nitrogen excretion, this is a question which the data are not yet sufficient to determine.

Finally there remains the case of malaria. In this instance, however, it was impossible to get normal control figures in the way



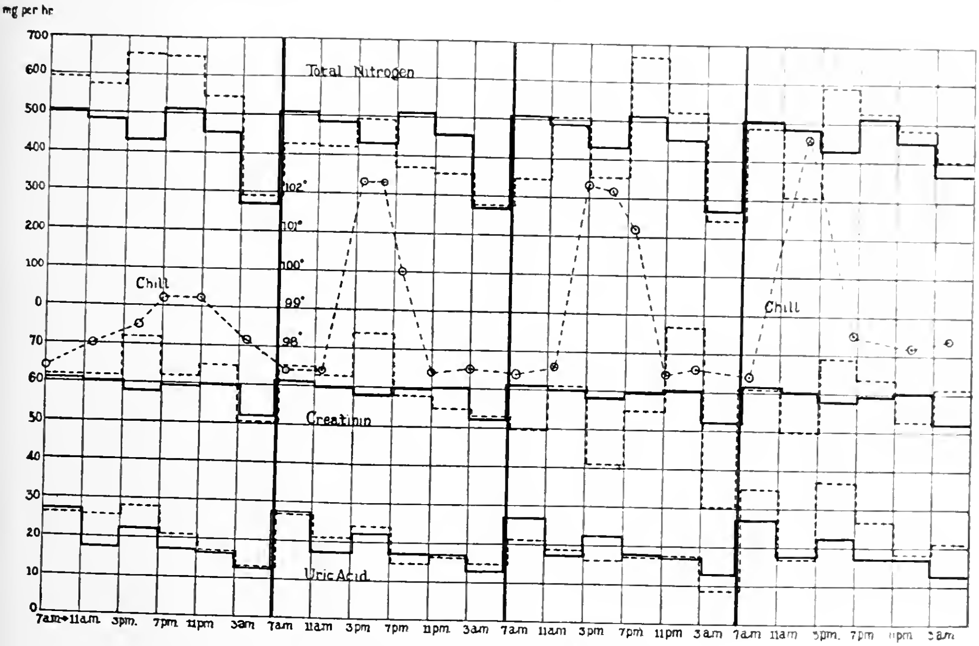
TEXT-FIG. 7. Case 7. Tuberculin, 3 doses as indicated in the text-figure, in a subject suffering from well marked tuberculosis.

TABLE III.

Malaria (Double Tertian).

Case 8. V. K.	Total nitrogen.		Uric acid.		Creatinin.		Maximum temperature F.	Remarks.
	Gm.	± per cent.	Gm.	± per cent.	Gm.	± per cent.		
1st dy......	13.2	+26	0.52	+16	1.49	+ 6	99.2°	Rigor.
2d dy......	9.4	−10	0.48	+ 7	1.47	+ 5	102.3°	
3d dy......	10.5	± 0	0.39	−13	1.26	−10	102.3°	
4th dy.....	11.2	+ 7	0.62	+38	1.43	+ 2	103.6°	Rigor.
5th dy.....	10.5		0.45		1.40		98.2°	Quinin.

The percentage variations are calculated with reference to the 5th day as standard, when quinin was given and there was no rise of temperature.



TEXT-FIG. 8. Case 8. Malaria.

in which this was done with the other cases. The patient had had several days of fever before he came under observation. Analyses were carried out on four days on which there was fever, on two of them preceded by rigors, and on a fifth day when administration of quinin prevented both rigor and fever. The figures obtained on the fifth day are charted as the continuous line and are given for com-

parison with those for each twenty-four-hour period on which fever occurred. As far as the available data go they show higher figures for uric acid and total nitrogen on the days on which there was rigor than on any of the others, and the fact that the effect of the reaction on the excretion of nitrogenous substances is different from that observed with the typhoid vaccine and also from that after tuberculin is perhaps in favor of the view that different pyretogenic toxins affect the excretion of nitrogen in different ways because the damage done to the cell protoplasm is different. But the lack of satisfactory controls makes the figures obtained from this case less significant (table III).

CONCLUSIONS.

The rise of temperature following typhoid vaccination is accompanied by a marked increase in the output of total nitrogen, creatinin, and especially of uric acid.

The reaction to tuberculin is slower, but is also accompanied by an increased output of nitrogenous substances, that is, however, less uniform and generally less pronounced.

In a case of malaria there was a tendency for the rise of temperature to be accompanied by an increased output of total nitrogen and less uniformly of creatinin; on two days on which the fever was preceded by rigor the output of uric acid was increased.

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LIVER FUNCTION AS INFLUENCED BY THE DUCTLESS GLANDS.*

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In recent publications[1,2] we have shown that valuable information concerning liver function and liver disease can be obtained by the intravenous injection of a solution of phenoltetrachlorphthalein. The drug is excreted through the bile by the activity of the hepatic epithelium and may be extracted from the feces and estimated with considerable accuracy. The phthalein excretion of normal dogs is quite constant, as is shown by the tables given below. The normal base line curve of phthalein excretion for an individual dog under uniform conditions is still more constant.

We have reported elsewhere[3] experiments that indicate that injury of the liver by means of specific poisons such as chloroform, phosphorus, and hydrazin causes a fall in the phthalein excreted in the feces. The fall in the phthalein curve is proportional to the amount of liver injury and in acute fatal poisoning may reach zero. Given a definite liver injury it is found that a definite amount of the phenoltetrachlorphthalein will appear in the urine, which is free from the drug under normal conditions. Moreover, injury of the liver by actual cautery will cause a definite drop in the phthalein curve, depending upon the amount of injury and inflammatory reaction.

Under various conditions in which the functional capacity of the liver cell is interfered with, the output of phthalein falls below

* Received for publication, June 23, 1914.

[1] Whipple, G. H., Mason, V. R., and Peightal, T. C., *Bull. Johns Hopkins Hosp.*, 1913, xxiv, 207.

[2] Whipple, G. H., Peightal, T. C., and Clark, A. H., *Bull. Johns Hopkins Hosp.*, 1913, xxiv, 343.

[3] Whipple, Peightal, and Clark, *loc. cit.*

normal, depending upon the extent of the derangement. For example, in passive congestion of the liver, either experimental or clinical, there will be periods of decreased phthalein output corresponding to the intensity of the stasis or breaks in cardiac activity. The Eck fistula (dog 12–2) provides evidence in the same direction. When the portal blood is shunted around the liver by this means we may not at first get a decrease in liver function, but with the development of liver atrophy and fatty degeneration, a distinct and sometimes a marked depression in the excretion curve will be noted.

It is clear that injury or any definite functional derangement of the liver is associated with a definite change in its excretion of phenoltetrachlorphthalein. Passive congestion and Eck fistula are of especial importance in connection with the experiments given below for they show that a change in nourishment of the liver cell leading to atrophy and perhaps fatty degeneration can be recognized by this functional test.

It is important to recall that shortly after repair of an injury by chloroform or phosphorus the liver may give evidence of hyperactivity and excrete more phthalein than under normal conditions. The same may occur after a small dose of a liver poison, which we may assume acts as an irritant to the liver cells. Young dogs and pups will, as a rule, show a slightly higher excretion than adult or old dogs.

All the evidence suggests strongly that a decrease in functional capacity of the liver is paralleled by a lowered phthalein output. May we not assume that a decrease in the phthalein output means a decrease in the functional capacity of the liver? We have studied the liver function in the pathological states brought about by removal of the various ductless glands. The liver function, as indicated by the phthalein excretion is definitely impaired when the body is suffering from pancreatic or adrenal insufficiency. The liver may show no histological evidence of this change in function, but a constant fall in the phthalein curve occurs which may return to normal if, in the case of partial adrenal extirpation, the remaining fragment undergoes an hypertrophy sufficient for the needs of the body.

After complete pancreatic extirpation there is a steady fall in

phthalein excretion which may fall as low as one third or one fifth normal. This fact speaks strongly for the idea that pancreatic activity is essential to proper liver function, which is the case with the adrenals; and it contradicts the idea that the pancreas tends to inhibit liver activity, while suggesting that the pancreas may be looked upon as having an accelerating influence on the liver. This may have some relation to recently proposed theories of diabetes.

Hypophysis extirpation may cause fluctuations in the excretion curve,—an initial fall and secondary rise. A definite fall occurs shortly before death and is noted before the appearance of subnormal temperatures which may supervene a day or so before death.

Thyroid extirpation causes no change in the excretion curve unless the parathyroids are interfered with. Perhaps in experiments of longer duration we may be able to demonstrate some change in liver function.

Parathyroid extirpation and tetany cause no fall in the phthalein excretion curve, but rather a slight rise above normal. In the last day of fatal tetany the liver function will be normal or slightly above normal, which is remarkable considering the prostration that is present at this time. Also given a falling curve due to pancreas extirpation one can produce a rise in output by parathyroidectomy and tetany, a fact which speaks in favor of the supposition that the parathyroid glands exert directly or indirectly inhibitory action upon the liver.

When we recall the amount of injury that must be done to the liver to cause a definite fall in the phthalein excretion curve, it is obvious what marked influence the adrenals and especially the pancreas must exert upon the liver. It is more than possible that this derangement in liver function may be an important factor in the symptom complex of ductless gland insufficiency.

METHOD.

The method employed has been described in detail in an earlier publication,[4] but some points will be reviewed here. The tetrachlorphthalein solution used for the injections was made up in 100 to 200 c.c. lots in 2.5 per cent. strength, as it was found that such a solution does not precipitate within a couple of weeks if kept in a dark place. The method of preparation has been previously

[4] Whipple, Peightal, and Clark, *loc. cit.*

described.[5] This solution for injection was standardized with great care against a known solution containing 10 mg. of tetrachlorphthalein to the liter. This insures the injection of 100 or 200 mg. at each injection and makes for constant excretion. The experiments were done in groups using from three to six dogs of which one normal animal served as a control. The solution was injected using a glass syringe and needle in the jugular vein. Injections were usually made about noon and on the following morning a purge of magnesium sulphate insured complete evacuation of the phthalein-containing feces. A second collection must be made to insure complete evacuation of the drug, but will usually be found to contain only a faint trace, provided the purgation has been satisfactory. It is important to clean the feces from the metabolism cages with great care, using a wash bottle and spatula. It is essential to add about 5 c.c. of alkali to the liter wash bottle, as this shows at once any traces of phthalein which otherwise might be overlooked and helps in the solution of the drug.

The method of extracting the phthalein from the feces may be outlined briefly as follows: The collected feces are diluted to one or two liters including 10 c.c. of strong sodium hydroxide (40 per cent.) and shaken in a machine until a uniform fluid is obtained. One tenth of this mixture is diluted to 500 c.c. with water and 4 to 5 c.c. of strong sodium hydroxide. Of this 500 c.c. solution, 100 c.c. are taken for precipitation with the calcium solution,—calcium chloride 20 per cent. To this 100 c.c. are added 5 to 10 c.c. of the calcium solution, followed by 5 c.c. of strong sodium hydrate and water up to 200 c.c. This effects a final dilution of one tenth of the feces to one liter, including the alkali and calcium solutions. This solution may be filtered at once and read against a standard phthalein solution containing 10 mg. per liter. The readings will be directly in per cent. if 100 mg. have been injected.

It will be noted that the phthalein excretion in normal dogs is constantly higher than in our last report. This is due to a more accurate method for standardizing the injection solution which in these experiments contained 100 mg. in each 5 c.c. In our earlier experiments the solutions were of constant strength, but the amount of phthalein was overestimated giving a lower excretion in the feces. All these dogs have been injected with the correctly standardized phthalein so that no fluctuations in the curve are attributable to this factor except some of the earlier observations in the first experiment (Eck fistula, dog 12-2) which were made during the year 1912-1913.

EXPERIMENTAL OBSERVATIONS.

In all operations the dogs were given morphia before operation and ether anesthesia during the operation, which in no instance lasted more than one hour. The usual aseptic surgical technique was employed and great care exercised in the postoperative treatment to insure a rapid return to normal if possible.

[5] Rowntree, L. G., Hurwitz, S. H., and Bloomfield, A. L., *Bull. Johns Hopkins Hosp.*, 1913, xxiv, 327.

ECK FISTULA OF EIGHTEEN MONTHS' DURATION.

Dog 12–2.—Black and tan mongrel, female.

Feb. 1, 1913. Ether anesthesia. Eck fistula produced as usual with ligature of portal vein at hilum of liver.

Feb. 3. Dog in good condition; weight 13½ pounds. The dog presented the usual picture seen in successful Eck fistula experiments, having occasional digestive upsets, but in general maintaining normal weight with gradual increase in strength and general activity. Phthalein excretion is shown in the accompanying table, and in part has been recorded elsewhere in an earlier report. The condition has been practically unchanged during the year succeeding the last observation. There were slight fluctuations in the weight curve, but the condition during this time has been uniformly excellent.

June 10, 1914, 12 M. Dog in excellent condition. Weight 16 pounds. Phthalein 0.1 gm. intravenously.

June 11. Urine contains a good deal of phthalein as usual. No feces.

June 12. Abundant fluid feces. Phthalein excretion 45 per cent. 4 P.M. Ether anesthesia and bleeding from femoral. Weight 15½ pounds.

Autopsy.—Performed at once. Thorax, heart, lungs, and spleen are all quite normal. Pancreas, kidneys, adrenals, thyroid, and parathyroid all normal. Stomach and intestinal tract normal. Eck fistula shows clean margins and an opening about 3 by 4 mm. The portal vein is completely obliterated at the site of the ligature, and there are no collaterals above this ligature. There are large collaterals in the neighborhood of the kidney, forming easy collateral circulation between the portal and lumbar veins. Liver after bleeding and removal of gall bladder weighs 189 gm. This is to be compared with the normal dog 13–45, weight 15¼ pounds, killed at the same time under the same conditions. Normal liver without gall bladder weighed 232 gm. The Eck fistula liver shows regular lobulation and definite evidence of fatty change. The bile passages are everywhere normal.

Microscopical Examination.—Liver shows a remarkable grade of fatty degeneration involving about all of the central two thirds of each lobule. The

DOG 12–2.

Eck Fistula of Eighteen Months' Duration.

Date.	Phthalein in gm.	Phthalein excreted, per cent.		Weight in pounds.	Remarks.
		Feces.	Urine.		
Feb. 1, 1913...	—	—	—	13.5	Eck fistula produced.
Apr. 1........	0.10	17	++	15.0	
Apr. 8........	0.10	20	0.25	16.0	
Apr. 15........	0.10	30	2.0	15.5	
May 26........	0.10	36	0.1	17.0	Dog improving.
June 17........	0.10	40	0.4	17.0	
July 10........	0.10	40	0.9	16.3	
Oct. 27........	0.10	34	1.0	15.7	Dog in good condition.
Apr. 27, 1914 ..	0.10	46	0.5	16.0	
June 10........	0.10	45	++	16.0	
June 12........	0.10			15.5	Autopsy.

liver cells contain large droplets of neutral fat and the nuclei stain sharply. There are no phagocytes nor any evidence of cell necrosis. The marginal third of each lobule is normal. Bile passages and canaliculi are normal. Other organs negative.

The preceding experiment (dog 12–2, Eck fistula) shows several points of interest. A dog can live in health for a year and a half with an Eck fistula. This dog had a liver smaller than a healthy normal dog of the same weight, and the Eck fistula liver was supplied by about one third the normal amount of blood, yet excreted about two thirds the normal amount of phthalein. This is a simple example of atrophy and slight fatty degeneration due to decreased or changed blood supply. The dog showed no clinical evidence of liver insufficiency and appeared to be in perfect health, yet this physiological test shows a uniformly subnormal output of phthalein. It must be kept in mind that the mere presence of an Eck fistula does not effect a drop in the phthalein curve which may remain normal for some time after the operation, but the drop in the curve follows when the liver atrophy develops. This comes out in experiments previously reported.[6] Another point of interest in this dog and others of similar type is that pregnancy will not develop. The dogs appear normal in all respects and may go in heat frequently and take the male repeatedly, but no pregnancy follows.

After the first few weeks when the animals are given plenty of bones and milk with no meat, these animals may be kept indefinitely on the usual mixed diet of cooked meat and bread cakes. They do not stand confinement in cages as well as normal animals, and exercise is needful. These observations bear on the question of operation in liver disease in man, and it seems that a suitable technique for the operation is all that is required before applying the Eck fistula operation successfully to human cases of cirrhosis in which the symptoms of portal obstruction are the dominant ones.

ADRENAL EXTIRPATION.

Dog 13-56.—Young male pup; weight 14 pounds.

Jan. 23, 1 P.M. Phthalein 0.1 gm. intravenously.

Jan. 24. Abundant feces. Phthalein excretion 68 per cent.

Jan. 27, 11 A.M. Ether anesthesia. Right adrenal removed completely. Left

[6] Whipple, Peightal, and Clark, *loc. cit.*

adrenal cut across, and the lower pole including about one third of the gland parenchyma left *in situ*. The upper two thirds were removed.

Jan. 28. Dog is quite weak, pulse fair, wound dry; weight 12¾ pounds. Phthalein 0.1 gm. intravenously.

Jan. 29. Diarrhea marked. Phthalein excretion 44 per cent.

Feb. 3 and 4. Dog improving and quite lively.

Feb. 7, 3 P.M. Dog appears normal; weight 13¼ pounds. Phthalein 0.1 gm. intravenously.

Feb. 8. Dog has diarrhea without any purgation. Phthalein excretion 52 per cent.

Feb. 10. Diarrhea continues.

Feb. 21, 12 M. Dog in good condition. Weight 13¾ pounds. Phthalein 0.1 gm. intravenously.

Feb. 22. Spontaneous diarrhea. Phthalein excretion 39 per cent.

Feb. 25. Soft feces and fluid stools persisted.

Apr. 8, 1 P.M. Dog in good condition; weight 16 pounds. Phthalein 0.1 gm. intravenously.

Apr. 10. Formed feces. Phthalein excretion 70 per cent.

May 1, 3 P.M. Dog is normal. Operation under ether anesthesia. Left adrenal shows very definite hypertrophy. The fragment is more than double its former size. Fragment cut transversely, the lower half removed and the upper half left intact.

Pancreas.—Both upper and lower arm of gland extirpated, but the parenchyma adherent to the duodenum was left undisturbed. Probably three fifths of pancreatic parenchyma were removed and the blood supply to the remainder left undisturbed.

May 2. Dog in good condition.

May 3, 12 M. Dog appears normal. Wound is dry. Weight 16 pounds. Phthalein 0.1 gm. intravenously.

May 4. No feces. Urine contains no sugar. 5 P.M. Fluid feces. Phthalein excretion 52 per cent.

May 6. Dog vomits occasionally, but has no diarrhea; weight 16¾ pounds. Phthalein 0.1 gm. intravenously.

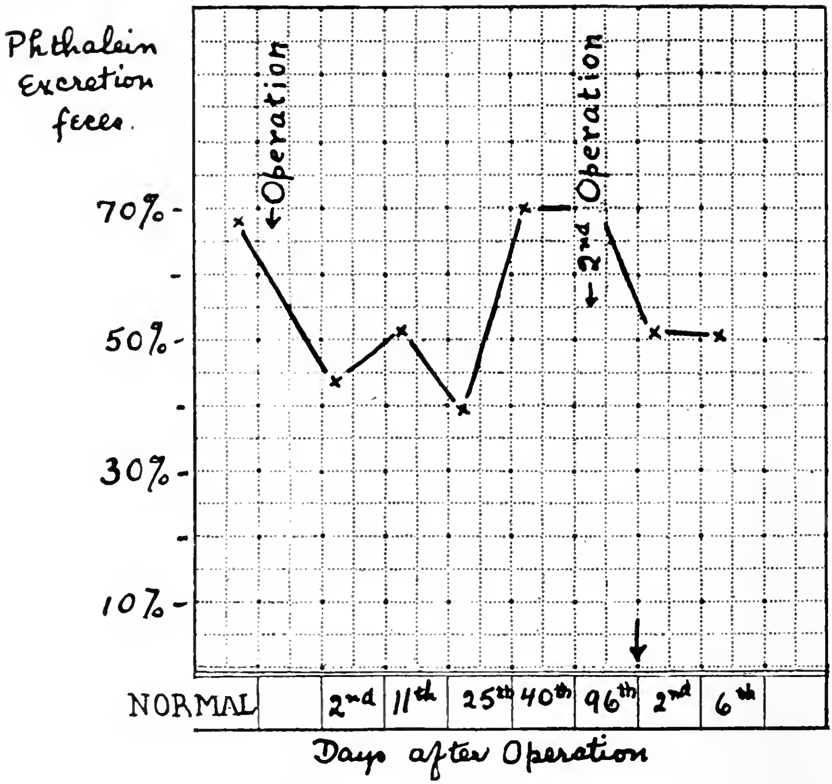
May 7. Abundant fluid feces. Phthalein excretion 51 per cent.

May 17. Dog appears quite sick and suffers from dyspnea. Temperature 35° C.

May 18. Found dead.

Autopsy.—Performed at once. The striking thing is the general anasarca. There is marked subcutaneous edema and accumulation of clear yellow fluid, about 300 c.c. in the peritoneal cavity, and almost the same amount in the thorax. The pericardial cavity is clear. Heart contains no clots. It shows dilatation and hypertrophy of the right side. Lungs show emphysema and small pulmonary thrombi. Spleen, kidneys, and intestinal tract normal. Liver is rather large, and lobules are conspicuous, due to edema. Pancreatic remnant looks normal. There are adhesions about the sites of operation. Adrenal fragment has increased in size to almost that found at the second operation. Cortex shows very marked hypertrophy. The cause of the general anasarca is quite obscure.

Microscopical Examination.—Adrenal gland removed at second operation shows the hypertrophy and increase in fat described by Crowe[7] in a recent publication. The adrenal fragment examined at autopsy shows the same picture together with a certain amount of necrosis, probably dependent upon interference with the blood supply. The parenchyma cells in places look much degenerated. There was undoubtedly a good deal of interference with the adrenal parenchyma and probably a marked insufficiency. The lungs show small patches of organizing pneumonia and induration. Pancreas and kidney are normal. Liver shows central congestion with slight atrophy and fatty degeneration. Some sections show definite central necrosis with thrombosis of adjacent capillaries.



TEXT-FIG. 1. Dog 13–56. Adrenal extirpation.

The preceding experiment (dog 13–56, text-figure 1) shows clearly the depression of liver function depending upon adrenal insufficiency. With hypertrophy of the remaining adrenal fragment

[7] Crowe, S. J., *Bull. Johns Hopkins Hosp.*, 1914, xxv (in press).

the liver function returns to normal, as would be expected. The second operation in which one half of the remaining adrenal fragment was removed was followed by a similar drop in phthalein excretion. At this second operation a portion of the pancreas was removed, but this procedure by itself would have no effect upon liver function. The cause of the anasarca is obscure, but may have been due in part to the changes in the pulmonary circulation causing hypertrophy of the right heart and more or less venous stasis.

The following experiment (dog 13–94) shows that a small adrenal fragment is more efficient in maintaining normal liver function when it is in connection with the sympathetic system by means of its nerves. Furthermore, a simple section of the large nerves going to this upper pole and no interference with the blood supply caused a depression in the phthalein curve when the first operation and removal of three fourths of the gland parenchyma had given no evidence of insufficiency.

ADRENAL EXTIRPATION. NERVE SECTION.

Dog 13–94.—Small fox-terrier, male; weight 13 pounds.

Mar. 20, 12 M. Phthalein 0.1 gm. intravenously.

Mar. 21. Abundant feces. Phthalein excretion 69 per cent.

Mar. 28, 11 A.M. Operation under ether anesthesia. Right adrenal removed completely. Left adrenal cut across leaving the upper two fifths of the gland intact, the lower three fifths being removed.

Mar. 29, 11 A.M. Dog in fair condition. Phthalein 0.1 gm. intravenously.

Mar. 30 and 31. Abundant feces. Total phthalein excretion 71 per cent.

Apr. 8. Dog very active; weight 13 pounds. Phthalein 0.1 gm. intravenously.

Apr. 9. Abundant feces. Phthalein excretion 74 per cent.

Apr. 25, 12 M. Operation under ether anesthesia. Left adrenal exposed with very little bleeding and no interference with the blood supply. The large nerve bundle to the upper pole was isolated and cut. The adrenal was handled very little and only this large nerve disturbed. Duration of operation about thirty minutes, which was about half the time required for the first operation, and there was much less handling of viscera during this second operation.

Apr. 26, 11 A.M. Dog is lively and appears normal in all respects. Weight 13¼ pounds. Phthalein 0.1 gm. intravenously.

Apr. 27 and 28. Abundant feces. Total phthalein excretion 59 per cent.

May 3, 12 M. Phthalein 0.1 gm. intravenously. Weight 13½ pounds.

May 4. Abundant feces. Phthalein excretion 72 per cent.

June 11. Dog normal. Weight 15 pounds. Ether anesthesia and bleeding from femoral.

Autopsy.—Performed at once. Thorax, heart, and lungs are normal. Thymus is large, milky, and soft. Testicles are of adult type. Lymph glands are not particularly enlarged, nor are the lymph follicles conspicuous in the intestine and spleen. The liver is quite normal. Pancreas, kidney, thyroid, and parathyroid are normal. Adrenal fragment has increased considerably in size. On section the cortex and medulla appear to be both involved in this hypertrophy.

Microscopical Examination.—Liver is normal. Thymus and lymphatic tissue show considerable hyperplasia. Adrenal shows the usual hyperplasia, especially of the cortex. Other organs normal.

DOG 13–94.

Adrenal Extirpation (Partial).

Date.	Phthalein in gm.	Phthalein excretion in per cent. in feces.	Weight in pounds.	Remarks.
Mar. 20.....	0.10	69	13	
Mar. 28.....	—	—		Adrenal extirpation.
Mar. 29.....	0.10	71		Good recovery.
Apr. 8.....	0.10	74	13	
Apr. 25.....				Sympathetic to adrenal fragment cut.
Apr. 26.....	0.10	59	13.3	Good recovery.
May 3.....	0.10	72	13.5	
June 11.....	—	—	—	Autopsy.

ADRENAL EXTIRPATION.

Dog A–5.—Black mongrel pup, female.

Dec. 8, 1913. Ether anesthesia and operation.[8] Right adrenal removed completely. Left adrenal in large part removed leaving small fragment, estimated at about one fifth of the gland.

Dec. 9, 12 M. Dog in fair condition; weight 10 pounds. Phthalein 0.09 gm. intravenously.

Dec. 10. Soft feces. Phthalein excretion 17 per cent.

Dec. 16, 1 P.M. Dog greatly improved and quite active, in spite of some loss of weight (9 pounds). Phthalein 0.1 gm. intravenously.

Dec. 17. Soft feces. Phthalein excretion 24 per cent.

Dec. 18. Much fluid feces. Phthalein negative. Dog appears prostrated. Pulse is very weak. Temperature 33.5° C. 4 P.M. Death.

Autopsy.—Gastritis, which may have been associated with purgation. Other organs normal. Adrenal fragment had undergone the usual hypertrophy. Liver and bile passages are quite normal.

ADRENAL EXTIRPATION.

Dog 13–107.—Strong mongrel pup, female; weight 19¾ pounds.

Apr. 8, 1 P.M. Phthalein 0.2 gm. intravenously.

Apr. 9. Abundant feces. Phthalein excretion 74 per cent.

Apr. 20. Ether anesthesia and operation. Right adrenal extirpated with the

[8] This operation was performed by Dr. S. J. Crowe.

exception of a tiny bit of parenchyma at the upper pole, less than one tenth of the substance of the gland. Blood supply much interfered with. Left adrenal extirpated leaving the lower one fourth *in situ*. The blood supply to this fragment was also somewhat interfered with. A good deal of bleeding and handling of viscera.

Apr. 21. Dog looks well, but pulse is weak and rapid. Diarrhea present. Temperature 37.5° C.

Apr. 22, 12 M. Dog in fair condition; weight 18 pounds. Temperature 38.2° C. Phthalein 0.2 gm. intravenously.

Apr. 23. Fluid feces. Phthalein excretion 28 per cent. Dog refuses food and given milk by stomach tube. 3 P.M. Death.

Autopsy.—Performed at once. The peritoneal cavity shows a little peritonitis at the site of operation, consisting mostly of fibrin, and there is no free fluid. Adrenal fragments show considerable edema and obvious necrosis, but some parenchyma seems normal. Liver and bile passages are normal. In this animal the adrenal insufficiency was close to the lowest possible limit.

The two preceding experiments (dogs A–5 and 13–107) show the remarkable depression of liver function that may be present in dogs that have almost the minimum necessary adrenal parenchyma. Both these dogs were close to this line of minimum adrenal tissue and the last dog might well have survived but for the peritonitis, which was too much for the animal in this condition of grave adrenal insufficiency. Such a drop in liver function to one third normal is found only in severe hepatic injury by poisons which, like chloroform, may destroy one half or more of the liver epithelium. This brings out the paralyzing effect of this adrenal insufficiency upon the liver cells which are quite normal histologically.

PANCREAS EXTIRPATION.

Dog 13–20.—Mongrel pup, male; weight 11½ pounds.

Apr. 19, 11 A.M. Phthalein 0.1 gm. intravenously.

Apr. 20. Phthalein excretion 59 per cent.

Apr. 23, 12 M. Ether anesthesia and operation. Pancreas completely extirpated, with clean dissection along the duodenum.

Apr. 24. Pup is active and lively. Weight 10¾ pounds. Phthalein 0.1 gm. intravenously.

Apr. 25. Abundant feces. Phthalein excretion 45 per cent.

Apr. 26, 11 A.M. Dog in good condition; weight 10½ pounds. Phthalein 0.1 gm. intravenously. This caused no appearance of phthalein in the urine.

Apr. 27. Abundant, soft feces. Phthalein excretion 24 per cent.

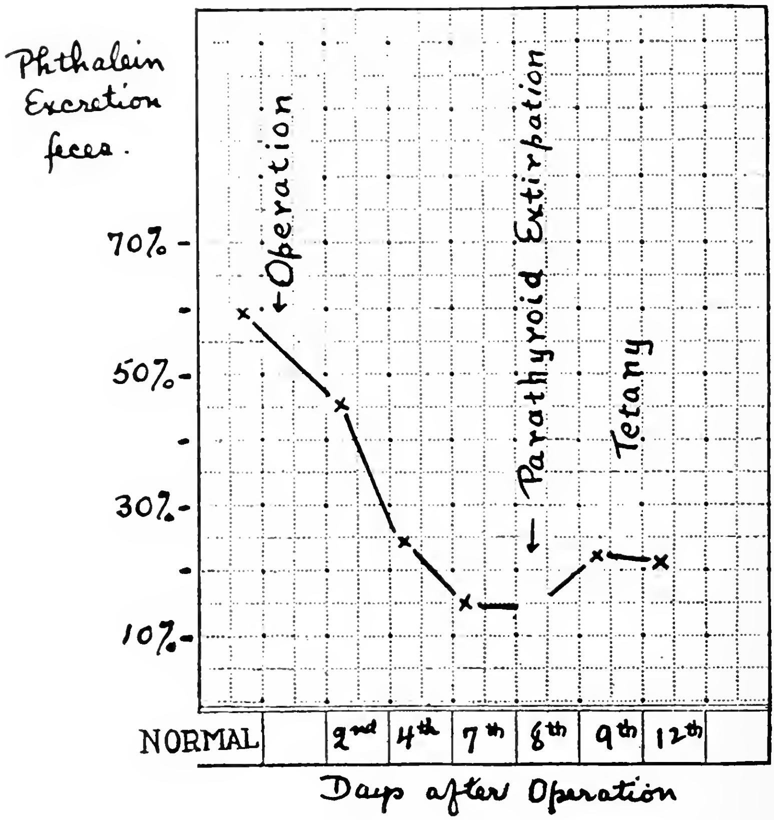
Apr. 28. Dog rather drowsy and eats but little.

Apr. 29. Urine contains a large amount of sugar. Dog is losing weight steadily (9 pounds). 11 A.M. Phthalein 0.1 gm. intravenously. 4 P.M. Urine contains phthalein 3.5 per cent.

Apr. 30. Much feces with diarrhea. Phthalein excretion 15 per cent. 3 P.M. Ether anesthesia for twenty minutes. Thyroid and parathyroid glands removed completely.

May 1. Dog refused food and given milk by stomach tube. Feces are fluid and slightly blood tinged. There is definite evidence of tetany, muscular twitchings being in evidence in the neck. 11 A.M. Phthalein 0.1 gm. intravenously.

May 2. Dog is dull and drowsy. Small amount of feces. Phthalein content 22 per cent.



TEXT-FIG. 2. Dog 13–20. Pancreas extirpation.

May 4, 9 A.M. Dog in poor condition. Temperature 39.5° C. Phthalein 0.1 gm. intravenously.

May 5, 9 A.M. Dog found dead. Some fluid feces in cage containing 17 per cent. phthalein. Urine contained no phthalein but some bile pigment.

Autopsy.—Performed at once. Intestinal contents as well as gall bladder analyzed for phthalein. 4 per cent. found, making a total phthalein output in about twenty hours of 21 per cent., which is about equal to or slightly more than

that of May 2. Lungs show a little bronchopneumonia in lower lobe. Adrenals show a slight atrophy of the cortex. Spleen is rather small, because of interference with the vascular supply at the time of operation on the pancreas. The liver is very fatty, suggesting the picture of phosphorus poisoning.

Microscopical Examination.—Liver cells about the portal spaces for a distance of one or two rows appear normal. Those in the center of the lobule contain great numbers of small fat droplets, but the nuclei are well preserved. No cell necrosis and no wandering cells in the capillaries. Adrenal cells contain a great amount of fat, but appear normal (text-figure 2).

PANCREAS EXTIRPATION.

Dog 13–98.—Yellow adult, male; weight 13½ pounds.

Mar. 19. Ether anesthesia and operation, with complete removal of pancreas, except a few small lobules close to the duodenum.

Mar. 20. Dog appears well. 12 M. Phthalein 0.1 gm. intravenously.

Mar. 21. Urine contains sugar, but no phthalein.

Mar. 22. Abundant feces, phthalein excretion 58 per cent. 12 M. Dog in good condition; weight 12½ pounds. Phthalein 0.1 gm. intravenously.

Mar. 23. Catheterized urine contains no phthalein but considerable sugar.

Mar. 24. Much feces. Total phthalein excretion 56 per cent. 4 P.M. Phthalein 0.1 gm. intravenously.

Mar. 25. Abundant fluid feces. Phthalein excretion 65 per cent.

Mar. 28. Condition unchanged; weight 13 pounds. 3 P.M. Phthalein 0.1 gm. intravenously.

Mar. 29. Abundant feces. Phthalein excretion 46 per cent.

Apr. 16. Dog in poor condition and much emaciated; weight 8½ pounds. 11 A.M. Phthalein 0.1 gm. intravenously. 4 P.M. Urine contains phthalein 1.2 per cent.

Apr. 17. Abundant feces. Phthalein excretion 44 per cent.

Apr. 18, 12 M. Ether anesthesia and operation with complete removal of thyroid and parathyroid glands. Anesthesia twenty minutes.

Apr. 19, 11 A.M. Dog shows signs of tetany; weight 9 pounds. Phthalein 0.1 gm. intravenously.

Apr. 20. Abundant feces. Phthalein output 50 per cent. Typical signs of tetany present.

Apr. 21, 12 M. Condition remains the same; weight 9 pounds. Temperature 37.2° C. Phthalein 0.1 gm. intravenously. 4 P.M. Urine contains phthalein 1.9 per cent.

Apr. 22. Dog in violent tetany. Abundant soapy feces. Phthalein excretion 55 per cent. 4 P.M. Death.

Autopsy.—Performed at once. Thorax, heart, and lungs negative. Total absence of subcutaneous and visceral fat. Kidneys normal. Adrenals are rather small, with atrophy of the cortex. Pancreas: there are a few tiny beads of parenchyma close to the duodenum and pylorus. The liver shows no evidence of fat. It looks rather atrophic. The lobules are regular and normal in color.

Microscopical Examination.—The adrenal glomerular zone is narrow. Fat

is inconspicuous in the cells. The liver shows slight central congestion. The liver cells show distinct atrophy, somewhat similar to that seen with the Eck fistula. Fatty change is not conspicuous. Phagocytic endothelial cells containing pigment are present in the capillaries.

DOG 13-98.

Pancreas Extirpation.

Date.	Phthalein in gm.	Phthalein excretion in per cent. in feces.	Weight in pounds.	Remarks.
Mar. 19.....	—	—	—	Pancreas extirpation.
Mar. 20.....	0.10	58	13.5	
Mar. 22.....	0.10	56	12.5	Sugar in urine.
Mar. 23.....	0.10	65	13.5	
Mar. 28.....	0.10	46	13.0	
Apr. 16.....	0.10	44	8.5	Dog emaciated and weak.
Apr. 18.....	—	—	—	Thyroid, parathyroid extirpation.
Apr. 19.....	0.10	50	9.0	Tetany present.
Apr. 21	0.10	55	9.0	Tetany marked.
Apr. 22.....	—	—	—	Death.

The two preceding experiments (dogs 13-20 and 13-98) show the same drop in phthalein output after pancreatic extirpation. The fall in the phthalein curve is much more marked when the pancreas removal is complete. Given this fall in phthalein curve due to pancreatic insufficiency, the onset of parathyroid tetany will cause a distinct rise in phthalein excretion. This experiment gives better evidence for the acceleration of liver function during parathyroid tetany than can be gotten in simple cases of tetany which will be discussed later.

PANCREAS EXTIRPATION.

Dog 13-104.—Mongrel black and tan, male.

Mar. 25, 4 P.M. Ether anesthesia and operation. Pancreas removed almost completely. A few tiny bits remain close to the duodenum.

Mar. 26. Dog in good condition.

Mar. 27. Dog doing well. Urine contains much sugar. Weight 14¼ pounds.

Mar. 28. Sugar abundant in urine. Weight 13 pounds. 3 P.M. Phthalein 0.1 gm. intravenously.

Mar. 29. Abundant soft feces. Phthalein excretion 47 per cent.

Apr. 2. Phthalein 0.1 gm. intravenously.

Apr. 3. Abundant feces. Phthalein output 56 per cent.

Apr. 8, 1 P.M. Dog is weak and emaciated. Weight 9½ pounds. 12 M. Phthalein 0.1 gm. intravenously. 6 P.M. Urine contains phthalein 2 per cent.

Apr. 9. Abundant feces, phthalein excretion 32 per cent.

Apr. 10, 2 P.M. Dog in poor condition and very weak. Ether anesthesia and bleeding.

Autopsy.—Performed at once. Thorax, heart, and lungs normal. Fat has completely disappeared. All viscera are normal except the pancreas and liver. One tiny nodule of the pancreas close to the duodenum remains. Dimensions 3 by 5 by 1 mm. Liver shows some focal yellow areas and the centers of all lobules are fatty. Elsewhere the parenchyma is brownish and translucent.

Microscopical Examination.—Liver resembles closely that of dog 13-98. The cells show a little atrophy and slight fatty degeneration. Large pigmented endothelial cells are found in the blood vessels. There are a few polymorphonuclears in the capillaries. No necroses.

THYROID AND PARATHYROID EXTIRPATION.

Dog 13-51.—Mongrel adult, male; weight 15½ pounds.

Jan. 17, 12 M. Ether anesthesia and operation; thyroid and parathyroids removed. At the end of the operation intravenous injection of phthalein 0.1 gm.

Jan. 18. Good recovery.

Jan. 19. Muscular tremors definite. Abundant feces. Phthalein output 35 per cent. 12 M. Muscular tremors are more marked; weight 16¾ pounds. Phthalein 0.1 gm. intravenously. 5 P.M. Convulsive seizures.

Jan. 20 and 21. Tremors present but less violent with milk diet. Total phthalein output 60 per cent.

Jan. 21, 6 P.M. Phthalein 0.1 gm. intravenously.

Jan. 23, 4 P.M. Total phthalein output 46 per cent. Delay in purgation, which was incomplete until Jan. 24, may explain this in part.

Jan. 24, 6 P.M. Phthalein 0.1 gm. intravenously. Weight 15½ pounds. Dog on milk diet.

Jan. 26. No feces.

Jan. 27. Much diarrhea. Total phthalein output 57 per cent. Dog in violent tetany and the slightest touch causes a violent spasmodic reaction. 5 P.M. Death.

Autopsy.—Performed at once. The autopsy showed nothing abnormal except venous engorgement of organs.

Microscopical Examination.—Liver normal.

THYROID AND PARATHYROID EXTIRPATION.

Dog 13-13.—Fox-terrier, male; weight 15¾ pounds.

Nov. 13, 12 M. Phthalein 0.08 gm. intravenously.

Nov. 15. Abundant feces. Phthalein output 45 per cent.

Dec. 23, 12 M. Dog in good condition. Ether anesthesia, and operation. Thyroid and parathyroids removed.

Dec. 24. Muscular tremors are present; weight 10½ pounds. 1 P.M. Phthalein 0.1 gm. intravenously.

Dec. 25. Abundant feces. Phthalein excretion 42 per cent.

Dec. 26. Phthalein 0.1 gm. intravenously.

Dec. 27. Muscular tremors very marked. Dog refuses food; weight 11½ pounds. Abundant feces. Phthalein output 68 per cent.

Dec. 28. Death.
Autopsy.—Normal organs throughout.

The two preceding experiments give definite evidence that there is no decrease in liver functional capacity even during the extreme prostration of fatal tetany. The last of the series (dog 13–13) suggests a definite rise above the normal base line. It must be recalled that the feces excretion of phenoltetrachlorphthalein may be very nearly the same whether the drug is injected intravenously or given by stomach tube. This shows how completely the drug is excreted by the normal liver and how little is lost in the transfer from the blood to the bile. One experiment may be cited as illustrative of this point.

Dog 13–125.—Young female pup; weight 14½ pounds.
May 6, 4 P.M. Phthalein 0.1 gm. intravenously.
May 8 and 9. Total phthalein excretion 57 per cent.
May 8, 12 M. Phthalein 0.1 gm. given with milk by stomach tube.
May 9, 12 M. Phthalein excretion 60 per cent.

This experiment shows how narrow the margin may be between the normal phthalein excretion and presumably total excretion into the alimentary tract. Other experiments may show a much greater difference.

One of the experiments given below (dog 13–36) gives evidence of a slight rise in phthalein excretion above normal in periods of mild tetany, as it is clear that this dog suffered at times from parathyroid insufficiency and died in tetany. Better and more convincing evidence that tetany causes an increase in phthalein liver excretion is found in the preceding experiments under pancreatic extirpation (dog 13–20 (text-figure 2) and dog 13–98). With a falling phthalein curve due to pancreatic insufficiency we see a definite rise caused by parathyroid tetany. This rise is noted, although the general condition of the dog is much less favorable than before the removal of the thyroid and parathyroid glands.

From all this data it seems safe to conclude that during tetany there is a tendency towards an increase in phthalein excretion by the liver, indicating an overstimulus and hyperactivity of the liver epithelium.

THYROID EXTIRPATION.

Dog 13-26.—Active adult, male; weight 20½ pounds.

Jan. 26, 3 P.M. Phthalein 0.2 gm. intravenously.

Jan. 27. Abundant feces. Phthalein output 65 per cent.

Mar. 21, 11 A.M. Operation with ether anesthesia. Extirpation of both thyroid lobes, one large parathyroid being left at the lower pole on the left side.

Mar. 22. Dog is well; weight 19 pounds. 12 M. Phthalein 0.2 gm. intravenously.

Mar. 23. Dog is normal. No feces.

Mar. 24. No signs of tetany. Abundant feces. Total output of phthalein 63 per cent.

Mar. 25. No muscular tremors. Weight 19¼ pounds. Appetite good. 12 M. Phthalein 0.2 gm. intravenously.

Mar. 26. Abundant feces. Phthalein excretion 63 per cent.

Apr. 16, 11 A.M. Dog in normal condition; weight 18¾ pounds. Phthalein 0.2 gm. intravenously.

Apr. 17. Abundant feces. Phthalein output 66 per cent.

June 8. Dog has been in good condition over the entire period since the operation. Weight 20½ pounds. 12 M. Phthalein 0.2 gm. intravenously.

June 9. Abundant feces. Phthalein excretion 67 per cent.

June 11. Ether anesthesia and bleeding.

Autopsy.—Performed at once. All the organs are perfectly normal. Neck shows no definite thyroid tissue. One large parathyroid is found and appears to be normal. Liver and bile passages normal.

Microscopical Examination.—Liver normal. Two tiny bits of thyroid tissue found in section. The acini contain little colloid and show a cubical type of epithelium. Parathyroid large and normal. Other organs normal.

DOG 13-26.

Thyroid Extirpation.

Date.	Phthalein in gm.	Phthalein excretion per cent. in feces.	Weight in pounds.	Remarks.
Jan. 26.....	0.20	65	—	
Mar. 21.....				Thyroid extirpation.
Mar. 22.....	0.20	63	19.0	No tetany.
Mar. 25.....	0.20	63	19.3	No tetany.
Apr. 16.....	0.20	66	18.7	No tetany.
June 8.....	0.20	67	20.5	Good condition.
June 11.....	—	—	—	Autopsy.

THYROID EXTIRPATION.

Dog 13-36.—Active young female; weight 21 pounds.

Jan. 19, 12 M. Phthalein 0.15 gm. intravenously.

Jan. 20. Abundant feces. Phthalein output 56 per cent.

Jan. 23. Chloroform 15 c.c. by stomach tube, causing slight intoxication.

Jan. 24. Dog will eat little food; weight 20¾ pounds. 6 P.M. Phthalein 0.2 gm. intravenously.

Jan 26. Abundant feces. Total phthalein output 19 per cent.

Jan. 28. Condition appears to be normal.

Feb. 7, 11 A.M. Ether anesthesia with removal of thyroid lobes. One parathyroid at left upper pole was left undisturbed.

Feb. 8. Dog appears well, except for slight muscular tremors. 10 A.M. Phthalein 0.2 gm. intravenously.

Feb. 9. Abundant feces. Total phthalein output 70 per cent.

Feb. 12. Dog active and hungry.

Feb. 15. Dog in good condition; weight 22 pounds. 11 A.M. Phthalein 0.2 gm. intravenously.

Feb. 16, 12 M. Abundant feces. Phthalein excretion 66 per cent.

Mar. 18. Dog in good condition, except for a little mange. Weight 21½ pounds. 4 P.M. Phthalein 0.2 gm. intravenously.

Mar. 19. Abundant feces. Phthalein output 52 per cent.

Apr. 16, 11 A.M. Dog in good condition. Weight 19¾ pounds. Phthalein 0.2 gm. intravenously.

Apr. 17. Soft feces. Phthalein output 62 per cent.

May 6, 4 P.M. Dog in good condition. Weight 21½ pounds. Phthalein 0.2 gm. intravenously.

May 7. No feces.

May 8. Abundant feces. Phthalein output 53 per cent.

June 1. Dog has developed violent tetany which resists all treatment.

June 2. Death.

Autopsy.—Organs showed advanced postmortem change.

DOG 13-36.

Thyroid and Partial Parathyroid Extirpation.

Date.	Phthalein in gm.	Phthalein excretion per cent. in feces.	Weight in pounds.	Remarks.
Jan. 20.....	0.15	57	21.0	
Jan. 23.....	—	—	—	Chloroform by stomach (15 c.c.)
Jan. 24.....	0.20	19	20.7	
Feb. 7.....	—	—	—	Thyroid and 3 parathyroids extirpated.
Feb. 8.....	0.20	70	18.5	Slight tetany.
Feb. 15.....	0.20	67	22.0	Good condition.
Mar. 18.....	0.20	53	21.5	
Apr. 16.....	0.20	62	19.7	
May 6.....	0.20	53	21.5	Good condition. No tetany.
June 1......	—	—	—	Violent tetany. Death.

The two preceding experiments (dogs 13-26 and 13-36) show that thyroid insufficiency has no effect upon the curve of phthalein liver excretion. During periods of tetany due to removal of too much parathyroid tissue the excretion curve may fluctuate. It is possible that longer periods of observation may bring out a change

in hepatic function with complete thyroid removal. Observations on human cases of exophthalmic goitre would be of interest in this connection.

HYPOPHYSIS EXTIRPATION.

Dog 13-79.—Active male pup; weight 15 pounds.

Feb. 18, 11 A.M. Phthalein 0.1 gm. intravenously.

Feb. 19. Abundant feces. Phthalein excretion 70 per cent.

Mar. 20, 12 M. Dog has practically recovered from distemper; weight 12½ pounds. Phthalein 0.1 gm. intravenously.

Mar. 22. Delay in purgation. Phthalein output 63 per cent.

Apr. 20. Ether anesthesia and operation.[9] Removal of hypophysis.

Apr. 21. Dog in good condition. Temperature 39.2° C. 12 M. Phthalein 0.1 gm. intravenously. Weight 15½ pounds.

Apr. 22. No feces.

Apr. 23. Soft feces. Phthalein output 52 per cent. Edema of head wound is marked. Temperature 39.2° C.

DOG 13-79.

Hypophysis Extirpation.

Date.	Phthalein in gm.	Phthalein excretion per cent. in feces.	Weight in pounds.	Remarks.
Feb. 18.....	0.10	70	15.0	Young pup.
Mar. 20.....	0.10	63	12.5	Recovering from distemper.
Apr. 20.....	—	—	—	Hypophysis extirpation (incomplete).
Apr. 21.....	0.10	52	15.5	Slight fever.
Apr. 24.....	0.10	57	15.0	Improving.
Apr. 27.....	0.10	67	14.7	Good condition.
Apr. 29.....	0.10	76	15.0	
May 3.....	0.11	57	15.0	Good condition.
May 8.....	0.10	52	16.5	
June 8.....	0.10	61	16.5	Good condition.
June 11.....	—	—	—	Autopsy. Remnant of anterior lobe.

Apr. 24, 12 M. Dog appears normal; weight 15 pounds. Phthalein 0.1 gm intravenously.

Apr. 25. Abundant feces. Phthalein output 57 per cent.

Apr. 27, 12 M. Condition unchanged; weight 14¾ pounds. Phthalein 0.1 gm. intravenously.

Apr. 28. Abundant feces. Phthalein output 67 per cent.

Apr. 29. Condition normal. 11 A.M. Phthalein 0.1 gm. intravenously.

Apr. 30. Abundant feces. Phthalein output 76 per cent.

May 3. Condition the same; weight 15 pounds. 12 M. Phthalein 0.11 gm.

May 4. Abundant feces. Phthalein output 57 per cent.

May 8. Good condition; weight 16½ pounds. 12 M. Phthalein 0.1 gm. intravenously.

[9] This operation was performed by Dr. A. P. Jones.

May 9. Abundant feces. Phthalein output 52 per cent.

June 8, 12 M. Good condition; weight 16½ pounds. Phthalein 0.1 gm. intravenously.

June 9. Abundant feces. Phthalein output 61 per cent.

June 11. Ether anesthesia and bleeding.

Autopsy.—Performed at once. Hypophysis absent except a small fragment of anterior lobe at the base of the sella turcica. All other organs appear normal in all respects. The subcutaneous fat is well preserved. Liver and bile passages are normal.

Microscopical Examination.—Liver is normal. Other organs are negative.

HYPOPHYSIS EXTIRPATION.

Dog 13-74.—Active young male; weight 18½ pounds.

Feb. 10, 10 A.M. Phthalein 0.1 gm. intravenously.

Feb. 11. Abundant feces. Phthalein output 66 per cent.

Feb. 17. Ether anesthesia and operation.[10] Complete removal of hypophysis.

Feb. 18. Condition good. 11 A.M. Phthalein 0.1 gm. intravenously.

Feb. 19. No feces.

Feb. 20. Abundant feces. Phthalein output 55 per cent.

Feb. 21. Dog improving; weight 20¼ pounds. 12 M. Phthalein 0.1 gm. intravenously.

Feb. 23. Abundant feces. Phthalein output 65 per cent.

Feb. 25, 11 A.M. Dog in good condition; weight 18½ pounds. Phthalein 0.1 gm. intravenously.

Feb. 26. Abundant feces. Phthalein output 69 per cent.

Feb. 28. Convulsions and coma, with almost complete absence of pulse, giving the appearance of death.

Mar. 1, 10 A.M. Dog is quite toxic. Temperature 36.8° C. Weight 17 pounds. Phthalein 0.1 gm. intravenously.

Mar. 2. Dog in semistupor. Temperature 36.7° C. No feces.

Mar. 3, 9.30 A.M. Pulse slow. Temperature 31.3° C. Weight 16 pounds. Thin, blood stained feces. Phthalein excretion 50 per cent. 2 P.M. Death.

DOG 13-74.

Hypophysis Extirpation.

Date.	Phthalein in gm.	Phthalein excretion per cent. in feces.	Weight in pounds.	Remarks.
Feb. 10.....	0.10	66	18.5	
Feb. 17.....	—	—	—	Hypophysis extirpated.
Feb. 18.....	0.10	55	18.7	
Feb. 21.....	0.10	65	20.3	Dog improving.
Feb. 25.....	0.10	69	18.5	Good condition.
Feb. 28.....	—	—	—	Convulsions and coma.
Mar. 1.....	0.10	50	17.0	Subnormal temperature.
Mar. 3.....	—	—	—	Death. Temperature 31.3° C.

[10] This operation was performed by Dr. S. J. Crowe.

Autopsy.—Performed at once. All organs are normal. Liver and bile passages are normal.

Microscopical Examination.—Spleen and lymph glands show slight hyperplasia of lymph follicles. Liver shows acute engorgement of centers of lobules.

HYPOPHYSIS EXTIRPATION.

Dog H-3.—Mongrel female; weight 17 pounds.

Jan. 26. Ether anesthesia and operation.[11] Complete removal of hypophysis.

Jan. 28. Dog in good condition; weight 17¼ pounds. 12 M. Phthalein 0.1 gm. intravenously.

Jan. 29. Abundant feces. Phthalein 41 per cent.

Jan. 31. Dog looks sick. Temperature 39.6° C. Weight 16½ pounds. Phthalein 0.1 gm. intravenously.

Feb. 1. Abundant feces. Phthalein output 62 per cent.

Feb. 3. Condition fair; weight 16 pounds. Temperature 39.8° C. Phthalein 0.1 gm. intravenously.

Feb. 4. Abundant feces. Total phthalein output 57 per cent.

Feb. 7. Dog is drowsy. 3 P.M. Phthalein 0.1 gm. intravenously. Weight 17¼ pounds.

Feb. 8. Abundant feces. Phthalein output 47 per cent.

Feb. 10. Dog developed definite signs of distemper.

Feb. 12. Dog is drowsy; weight 15½ pounds. 5 P.M. Phthalein 0.1 gm. intravenously.

Feb. 13. No feces.

Feb. 14. Abundant feces. Phthalein excretion 46 per cent.

DOG H-3.

Hypophysis Extirpation.

Date.	Phthalein in gm.	Phthalein excretion per cent. in feces.	Weight in pounds.	Remarks.
Jan. 26.....				Hypophysis removal.
Jan. 28.....	0.10	41	17.3	Temperature 39.4° C.
Jan. 31.....	0.10	62	16.5	Temperature 39.6° C.
Feb. 3.....	0.10	57	16.0	
Feb. 7.....	0.10	47	17.3	Dog very drowsy.
Feb. 12.....	0.10	46	15.5	Distemper mild.
Feb. 16.....	0.10	40	14.0	Convulsions.
Feb. 17.....	—	—	—	Death.

Feb. 16. Dog in convulsions. Temperature 40.9° C. Weight 14 pounds. 3.30 P.M. Phthalein 0.1 gm. intravenously.

Feb. 17. Dog in stupor; pulse weak. 10.30 A.M. Death. Phthalein in cage and from intestinal contents combined, 40 per cent.

Autopsy.—Performed at once. Lungs show ecchymoses and some small patches of bronchopneumonia. Pancreas shows fat necroses with hemorrhage.

[11] This operation was performed by Dr. A. P. Jones.

an example of spontaneous hemorrhagic pancreatitis. Kidneys show small gray nodules in the cortex. Intestinal tract negative. Liver is rather pale and fatty.

Microscopical Examination.—Pancreatitis is of considerable age, showing a good deal of scar tissue throughout the gland, although there are some relatively fresh necroses of pancreas parenchyma and fat. The lungs show atelectasis and some bronchopneumonia. The kidneys show accumulations of mononuclear wandering cells in some parts of the cortex. Parenchyma is normal. There is slight atrophy of the liver cells in the center of the lobule. Otherwise normal.

The three preceding experiments are uniform in all respects. Complete removal of the hypophysis will cause little change in the liver function, as indicated by phthalein excretion. There is an initial drop in the phthalein excretion following the operation, and this cannot be attributed to the anesthetic. Possibly the cerebral edema and trauma may account for it. This is followed by a recovery to normal or even a little above normal. Just before the fall in body temperature, which usually precedes death in these dogs, there is a definite impairment of liver function. In the experiment (dog 13–79) in which a bit of the anterior lobe remained, one sees minor fluctuations in the phthalein curve, which may be dependent in part on the hypophysis insufficiency which was undoubtedly present.

SUMMARY.

When phenoltetrachlorphthalein is injected intravenously, it is eliminated from the body in the bile through the activity of the hepatic epithelium. The feces may be collected after purgation and the phthalein extracted and estimated against a standard solution. The estimation of phthalein can be done with accuracy in a suitable colorimeter and the elimination in normal dogs is quite constant.

Given a definite liver injury by means of poisons (chloroform, phosphorus), the amount of phthalein excreted will be diminished and the fall in output will be proportional to the amount of injury. With an acute fatal poisoning the curve may fall to zero.

Under certain conditions of vascular interference the liver phthalein may show a decreased output; in passive congestion of the liver and with the Eck fistula the liver output may fall considerably below normal.

Known disturbances of the liver function due to parenchymatous

injury or vascular disturbances are indicated by a fall in the phthalein excretion curve. Conversely it may be claimed that a drop in phthalein excretion may indicate a decrease in the functional capacity of the liver even if there be no detectable histological changes.

Adrenal insufficiency produced by extirpation of three fourths or more of the gland tissue will be associated with a drop in liver phthalein excretion. With hypertrophy of the adrenal fragment the excretion comes back to normal, but may fall again when more adrenal tissue is removed (text-figure 1).

Pancreatic insufficiency causes a progressive fall in the phthalein excretion indicating a grave lowering of the functional capacity of the liver (text-figure 2). This fact has a direct bearing on the question of diabetes.

Parathyroid insufficiency with tetany causes no decrease in phthalein output, but at times a rise above normal. This comes out best when the phthalein curve is low following pancreas extirpation. Parathyroid tetany may cause hyperactivity on the part of the liver cells.

Thyroid insufficiency produces no change in the uniform curve of phthalein excretion.

Hypophysis insufficiency shows an initial fall in the curve, followed by a return to normal and a final drop in the last few days before death.

These experiments supply evidence to the effect that the liver is very much concerned in the derangement that follows the removal of the ductless glands. Hence it seems probable that this disturbance of the liver function may be an important factor in the general symptom complex of ductless gland insufficiency.

In conclusion we wish to express our appreciation to Dr. S. J. Crowe and Dr. A. P. Jones for assistance rendered in performing some of the operations for gland removal.

THE RELATION OF BACTERIOLYSIS TO PROTEOLYSIS.

Studies on Ferment Action. XVI.*

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The relation of the proteolytic ferments of serum to immunity reactions, particularly to bacteriolysis, has interested several investigators, and in view of the recent work of Abderhalden and his collaborators it merits attention quite apart from a theoretical consideration. The study of possible proteolytic cleavage during bacteriolysis, has, because of the manifest technical difficulties entailed by the accurate determination of nitrogen in the small amounts available, been limited to rather general statements, based, in most instances, on experiments carried out with the Sörensen method. This method, being an index of the total cleavage of the protein molecule, gives no information of the change from coagulable to non-coagulable forms, and is therefore of use in estimating peptolytic or ereptic enzyme activity rather than proteolytic changes. For a like reason it represents the splitting of the protein bodies to non-toxic fragments in contradistinction to the splitting of the whole non-toxic protein molecule to the higher non-coagulable but toxic forms (proteoses). Since the microchemical methods devised by Bang (1) and Folin (2) were described there are available no experiments dealing with this subject, and we have therefore undertaken the following experiments using the Folin method, which lends itself admirably to work of this kind.

The bactericidal and bacteriolytic property of fresh serum due to the action of complement and amboceptor has been assumed, more particularly by French and German writers, to be enzymotic in character and in the nature of a protease. There is, however, no experimental basis for this idea; indeed the fact that complement

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action is not identical with a protease action can be demonstrated in a very simple experiment. When chloroform is added to fresh serum and thoroughly mixed, the complement is very rapidly destroyed; the serum protease, however, first becomes active under these conditions (Delezenne and Pozerski (3)). The activation under the influence of chloroform or other lipoid solvents is due to the fact that the antiferment is removed in this way. There should therefore be no confusion as to the non-identity of these serum constituents. If complement activity is enzymotic in character, we should rather seek to identify it with the lipases or allied enzymes. Thus the objection made that complement acts in direct proportion to the concentration, and is therefore unlike other ferments, fails in view of the fact that serum esterase acts in a like manner (Loevenhart (4)). And the ease with which lipoid solvents inactivate the complement finds an analogy in plant lipases which are either destroyed by lipoidal solvents or are in such intimate relation to the lipoids that they are taken up with them into the solvent. The substrate offered for complement action—red blood cells, animal cells, bacteria—is lipoidal in nature, the potentially lipoidal character of the limiting membranes of the corpuscles and animal cells being well established and rendered more than probable for bacterial cells. The fact that powerful proteolytic enzymes are without effect on such substrata is almost conclusive evidence that the surfaces of these substrata are not protein in nature. An observation of Metchnikoff (5) is significant in this connection. Metchnikoff, noting that the intestinal tract of certain insects (moths) was free from bacteria, an exception to the condition found in other insects, concluded that these insects must secrete a powerful fat-splitting enzyme to enable them to utilize waxes and fats readily, and surmised some connection between the character of this intestinal secretion and the freedom from bacteria.

EXPERIMENTAL.

The experiments, designed to determine the amount of proteolysis occurring during bacteriolysis, were carried out in the following manner. The organisms used, *Bacillus coli, Bacillus typhosus,* and staphylococci, were grown on agar in large bottles. They were

washed repeatedly with salt solution and used as fresh emulsions, or dried at low temperature and made up in a 1 per cent. emulsion as used. In either case the total nitrogen and the total non-coagulable nitrogen per cubic centimeter of the emulsion were determined for several samples. Immune sera were obtained by bleeding rabbits when the blood contained the greatest concentration of immune bodies. The immune sera so obtained were kept without preservatives on ice and were inactive. Fresh guinea pig serum was used as complement.

One cubic centimeter of the bacterial emulsion was measured into small centrifuge tubes, the various sera were then added, and digestion was permitted at 37° C. under toluol for varying periods of time. The tubes were then thoroughly centrifuged until the supernatant fluid was clear. The fluid was carefully pipetted from the bacteria, these were washed once with salt solution, and the fluid was added to that already withdrawn. The total nitrogen of the bacterial rest was then determined and also the total non-coagulable nitrogen of the supernatant fluid. The difference between this latter figure and the amount of total non-coagulable nitrogen in the serum controls gives the amount of nitrogen which has autolyzed, *i. e.,* the bacterial nitrogen digestion. The difference between the total nitrogen of the bacteria originally introduced and the amount recovered indicates the amount of bacteriolysis.

In using the relatively large amounts of organisms necessitated by the character of the work, the amount of serum is by comparison small, but the bacteriolytic effect is in most cases quite evident by the greater loss of bacterial nitrogen in the tubes containing the immune serum and complement.

The work has been carried out in duplicate and in some instances four or more determinations were made on corresponding mixtures. For the sake of brevity the protocols have been condensed and show only single determinations. The variations were seldom greater than a few hundredths of a milligram. The figures indicated in the protocols express nitrogen in milligrams.

In numerous experiments the bacterial rests remaining after serum treatment were digested by trypsin after determinations on similar mixtures showed the amount of substrate remaining. The

PROTOCOL I.

Tube No.	Action of immune serum and complement on bacteria and relation of resulting lysis to proteolysis.								Action of trypsin on bacteria following immune serum and complement action.					
	Typhoid bacilli.	Colon bacilli.	Colon immune serum.	Complement.	Bacterial nitrogen recovered.	Loss in bacterial nitrogen.	Total non-coagulable nitrogen in supernatant fluid.	Gain in non-coagulable nitrogen in supernatant fluid.	Typhoid bacilli.	Colon bacilli.	Trypsin.	Bacterial nitrogen (substrate).	Nitrogen digestion.	Digestion.
1	1.0 c.c.	Total nitrogen = 1.65 mg. per c.c.					Total non-coagulable nitrogen = 0.25 mg. per c.c.		......	......	......	......	......	......
2	......	1.0 c.c. Total nitrogen = 2.80 mg. per c.c.					Total non-coagulable nitrogen = 0.38 mg. per c.c.		......	......	......	......	......	......
3	1.0 c.c.	......	......	......	1.4 mg.	0.25 mg.	0.2 mg.	...... Autolysis in salt solution	......	......	......	......	......	......
4	......	1.0 c.c.	......	......	2.46 mg.	0.34 mg.	0.3 mg.	......	......	......	......	......	......	......
5	1.0 c.c.	......	......	......	1.51 mg.	0.14 mg.	0.2 mg. Total nitrogen in supernatant fluid		1.0 c.c.	......	0.1 c.c.	1.45 mg.	0.78 mg.	49%
6	......	1.0 c.c.	......	......	2.34 mg.	0.46 mg.	0.53 mg.		......	1.0 c.c.	0.1 c.c.	2.4 mg.	0.75 mg.	31%
7	1.0 c.c.	......	1.0 c.c.	2.0 c.c.	1.25 mg.	0.40 mg.	0.87(−0.625) mg.	0.24 mg. Typhoid bacilli, colon immune serum complement	1.0 c.c.	......	0.1 c.c.	1.25 mg.	0.48 mg.	38%
8	1.0 c.c.	......	0.1 c.c.	2.0 c.c.	1.25 mg.	0.40 mg.	0.66(−0.437) mg.	0.22 mg.	1.0 c.c.	......	0.1 c.c.	1.25 mg.	0.50 mg.	40%
9	......	1.0 c.c.	1.0 c.c.	2.0 c.c.	2.10 mg.	0.7 mg.	1.0 (−0.625) mg.	0.375 mg. Colon bacilli, colon immune serum complement	......	1.0 c.c.	0.1 c.c.	2.10 mg.	0.77 mg.	34%
10	......	1.0 c.c.	0.1 c.c.	2.0 c.c.	2.10 mg.	0.7 mg.	0.8 (−0.437) mg.	0.363 mg.	......	1.0 c.c.	0.1 c.c.	2.10 mg.	0.508 mg.	24%
11	1.0 c.c.	......	......	2.0 c.c.	1.1 mg.	0.55 mg.	0.61(−0.417) mg.	0.2 mg. Complement	1.0 c.c.	......	0.1 c.c.	1.1 mg.	0.45 mg.	40%
12	......	1.0 c.c.	......	2.0 c.c.	2.34 mg.	0.46 mg.	0.8 (−0.417) mg.	0.383 mg.	......	1.0 c.c.	0.1 c.c.	2.34 mg.	0.57 mg.	24%
13	1.0 c.c.	......	1.0 c.c.	......	1.2 mg.	0.45 mg.	0.5 (−0.207) mg.	0.293 mg. Immune serum	1.0 c.c.	......	0.1 c.c.	1.2 mg.	0.55 mg.	45%
14	......	1.0 c.c.	1.0 c.c.	......	2.4 mg.	0.4 mg.	0.56(−0.207) mg.	0.353 mg.	......	1.0 c.c.	0.1 c.c.	2.4 mg.	1.0 mg.	40%
15	......	......	1.0 c.c.	......	......	......	0.207 mg.	...... Non-coagulable nitrogen in serum controls	......	......	......	......	......	......
16	......	......	......	2.0 c.c.	......	......	0.417 mg.	......	......	......	......	......	......	......

per cent. of digestion was determined by carefully acidifying and boiling and filtering through kaolinized hard paper filters to remove the coagulated protein. The solution of trypsin used was prepared each time from a dried preparation and used in such dilution that 0.1 of a cubic centimeter of the solution would digest two cubic centimeters of a 1 per cent. casein solution in two hours. Protocol I will serve as an example of such an experiment.

PROTOCOL I—A.

Autolysis in Physiological Salt Solution. Relation of Solution of Bacteria to Autolysis.

Tube No.	Typhoid bacilli.	Colon bacilli.	Total nitrogen in supernatant fluid after 24 hrs.	Total nitrogen in supernatant fluid after 48 hrs.	Total non-coagulable nitrogen in supernatant fluid after 24 hrs.	Total non-coagulable nitrogen in supernatant fluid after 48 hrs.	
1	1.0 c.c.		0.37 mg.				Solution.
2	1.0 c.c.			0.6 mg.			
3	1.0 c.c.				0.2 mg.		Autolysis.
4	1.0 c.c.					0.2 mg.	
5		1.0 c.c.	0.57 mg.				Solution.
6		1.0 c.c.		0.8 mg.			
7		1.0 c.c.			0.35 mg.		Autolysis.
8		1.0 c.c.				0.5 mg.	

Fresh typhoid and colon bacilli were used. The typhoid emulsion contained 1.65 milligrams of total nitrogen and 0.25 of a milligram of total non-coagulable nitrogen per cubic centimeter. The colon emulsion contained 2.80 milligrams of total nitrogen and 0.38 of a milligram of total non-coagulable nitrogen per cubic centimeter. Bacteria were permitted to autolyze in salt solution over night (tubes 3 to 6); with immune colon serum and fresh complement (tubes 7 to 10); with complement alone (tubes 11 and 12); and with immune colon serum alone (tubes 13 and 14). The total non-coagulable nitrogen in the sera used is shown in tubes 14 and 15, and these amounts, viz., $0.417 + 0.207 = 0.624$ of a milligram, $0.417 + 0.02 = 0.437$ of a milligram are, of course, to be deducted from the digestion mixtures. A parallel series of tubes was prepared, serum action permitted for an equal length of time, the bacteria washed and subjected to tryptic digestion for six hours.

It is apparent in observing the results obtained that the greatest

loss of bacterial nitrogen has occurred in the mixtures with colon bacilli, colon immune serum, and complement, indicating a marked lysis of these bacteria. There is, however, no corresponding increase in non-coagulable nitrogen obtained in the supernatant fluid, the amount so recovered in the 1.0 and 0.1 cubic centimeter dilutions of immune serum being respectively 0.375 of a milligram and 0.363 of a milligram, corresponding very well with the amount of non-coagulable nitrogen introduced originally with the bacteria, 0.38 of a milligram, and representing only a trace more than the amount recovered in the salt solution control (tube 4). There is obviously no relation here between the amount of lysis and the actual proteolysis. It is interesting to note that the greatest lysis of typhoid bacilli occurred in the mixture containing complement alone, although here, too, there is no corresponding increase in non-coagulable nitrogen. When the bacterial residues are digested by trypsin the percentage of digestion is found to be considerably less in all the tubes treated with serum than in the untreated controls (tubes 5 and 6), with the exception of the bacteria treated with immune serum (tubes 9 and 14). We have previously shown (6) that bacteria treated with normal serum become more resistant to tryptic digestion, because of their adsorption of serum antitrypsin.

In protocol I-A is shown the rate of solution and of autolysis of

PROTOCOL II.

Action of Typhoid Immune Serum and Complement on Typhoid Bacilli. No Demonstrable Proteolysis.

Tube No.	Typhoid bacilli.	Immune serum.	Complement.	Bacterial nitrogen recovered.	Loss.	Total nitrogen in supernatant fluid.	Gain in total nitrogen.	Total non-coagulable nitrogen in supernatant fluid.	Gain in non-coagulable nitrogen.
1	1.0 c.c.	Total nitrogen = 1.1 mg., total non-coagulable nitrogen = 0.16 mg. per c.c.							
2	1.0 c.c.	Autolysis in sodium carbonate solution		0.66 mg.	0.44 mg.	0.43 mg.	....	0.17 mg.	....
3	1.0 c.c.	0.1 c.c.	1.0 c.c.	0.83 mg.	0.27 mg.	7 (−6.7) mg.	0.3 mg.	....	....
4	1.0 c.c.	0.1 c.c.	1.0 c.c.	0.83 mg.	0.27 mg.	....	....	0.55 (−0.39) mg.	0.16 mg.
5	......	0.1 c.c.	1.0 c.c.	....	....	6.7 mg.	....	....	....
6	......	0.1 c.c.	1.0 c.c.	....	....	....	....	0.39 mg.	....

these organisms when kept in salt solution at 37° C. under toluol. While solution has occurred in both cases, the autolysis is not equally rapid; indeed it did not occur at all in the typhoid emulsion in this case.

In protocol II a typhoid system was used, with fresh typhoid bacilli and typhoid immune serum. The total nitrogen per cubic centimeter was 1.1 milligrams; the total non-coagulable nitrogen was 0.16 of a milligram per cubic centimeter. Mixtures were made as usual, the reaction in this case being made distinctly alkaline to phenolphthalein, the control being placed in a sodium carbonate solution of equal alkalinity. The control lost 0.44 of a milligram of bacterial nitrogen, 0.43 of a milligram being recovered from the supernatant fluid, of which 0.17 of a milligram was non-coagulable, indicating a lysis without proteolysis in the control. It will be recalled that autolytic enzymes in general act best in a slightly acid medium. In the immune serum (tubes 3 and 4) there has been less lysis than in the alkaline control (loss of 0.27 of a milligram), and while this amount was recovered in the increase in total nitrogen in the supernatant fluid (0.3 of a milligram), the non-coagulable nitrogen had not increased (0.16 of a milligram). It is quite apparent that the solution of the bacteria, due in one case to the alkalinity of the fluid (tube 2), in the other to the lytic effect of the serum complement and immune body (tubes 3 and 4), is not accompanied by proteolysis.

If we permit the action of the immune serum and complement on the bacteria for a shorter period of time (4 hours at 37° C.),

PROTOCOL III.

Action of Typhoid Immune Serum and Complement on Typhoid Bacteria. Absence of Proteolysis after 4 Hours' Incubation.

Tube No.	Typhoid bacilli.	Typhoid immune serum.	Complement.		Bacterial nitrogen recovered.	Loss.	Gain in non-coagulable nitrogen
1	1.0 c.c.	Total nitrogen = 1.42 mg., total non-coagulable nitrogen = 0.2 mg. per c.c.					
2	1.0 c.c.	0.0 c.c.	0.0 c.c.	Autolysis	1.2 mg.	0.22 mg.	0.15 mg.
3	1.0 c.c.	0.0 c.c.	1.0 c.c.	....	1.2 mg.	0.22 mg.	0.135 mg.
4	1.0 c.c.	1.0 c.c.	1.0 c.c.	....	1.28 mg.	0.14 mg.	0.07 mg.
5	1.0 c.c.	0.1 c.c.	1.0 c.c.	....	1.25 mg.	0.17 mg.	0.13 mg.
6	1.0 c.c.	0.01 c.c.	1.0 c.c.	....	1.28 mg.	0.14 mg.	0.125 mg.

which is ample for the bactericidal effect of the serum even if not for actual bacteriolysis, there is practically no loss in bacterial nitrogen and no increase in non-coagulable nitrogen. As a matter of fact, there is much less splitting in the tube containing the greatest amount of antibody (tube 4, protocol III).

The fact that complement alone has no appreciable proteolytic effect even when bactericidal is shown clearly in protocol IV, in

PROTOCOL IV.

Effect of Complement on Colon Bacilli. No Resulting Proteolysis.

Tube No.	Colon bacilli.	Complement.	Reaction.	Bacterial nitrogen recovered.	Loss	Total nitrogen recovered in supernatant fluid.	Total non-coagulable nitrogen in supernatant fluid.	Gain in non-coagulable nitrogen.
1	1.0 c.c.	Total nitrogen	= 0.9 mg.	per c.c.		....	....	....
2	1.0 c.c.	......	Acid	0.39 mg.	0.51 mg.	0.5 mg.	....	0.17 mg.
3	1.0 c.c.	......	Alkaline	0.39 mg.	0.51 mg.	0.5 mg.	....	0.15 mg.
4	1.0 c.c.	1.0 c.c.	Acid	0.56 mg.	0.34 mg.	....	0.45(−0.23) mg.	0.22 mg.
5	1.0 c.c.	1.0 c.c.	Alkaline	0.44 mg.	0.44 mg.	....	0.3 (−0.23) mg.	0.07 mg.
6	......	1.0 c.c.	....	....	....	....	0.23 mg.	....

which dried colon bacilli were used. Dried organisms are, as a rule, more easily digested by trypsin than freshly killed organisms, possibly due to alterations in the physical state of the external limiting membrane of the organisms. The bacillary emulsion contained 0.9 of a milligram of total nitrogen per cubic centimeter. When permitted to autolyze in salt solution, both acid and alkaline in reaction, the solution of the organisms was quite marked, only 0.39 of a milligram remaining after twenty-four hours. The total dissolved nitrogen recovered from the supernatant fluid was 0.5 of a milligram, of which 0.17 of a milligram was non-coagulable. When treated with fresh guinea pig complement the lysis was not so marked, being greatest in the tubes with an alkaline reaction; the proteolysis, on the other hand, was greatest in an acid reaction. The increase of 0.05 of a milligram over the amount in the control has, however, no significance, for such conditions never occur in the living animal.

In studying the effect of varying amounts of the antibody on the bacteria and their consequent resistance to tryptic digestion, we have at various times observed that bacteria treated with the largest amount of antibody became more resistant to the action of trypsin,

thus in a measure simulating the Neisser-Wechsberg phenomenon. Such an experiment is shown in protocol V.

PROTOCOL V.

Effect of Tryptic Digestion on Organisms Treated with Varying Amounts of Specific and Non-Specific Immune Serum and Complement.

Tube No.	Typhoid bacilli.	Staphylococci.	Typhoid immune serum.	Complement.	Trypsin.	Tryptic digestion.	Increase.
1	1.0 c.c.	....	Total nitrogen = 1.4 mg., total non-coagulable nitrogen = 0.07 mg. per c.c.				
2	....	1.0 c.c.	Total nitrogen = 0.715 mg., total non-coagulable nitrogen = 0.16 mg. per c.c.				
3	1.0 c.c.	....	....	....	0.1 c.c.	0.625 mg.	....
4	....	1.0 c.c.	....	....	0.1 c.c.	0.15 mg.	....
5	1.0 c.c.	....	3.0 c.c.	0.3 c.c.	0.1 c.c.	0.54 mg.	−6%
6	1.0 c.c.	....	1.0 c.c.	0.1 c.c.	0.1 c.c.	0.625 mg.	....
7	1.0 c.c.	....	0.1 c.c.	0.1 c.c.	0.1 c.c.	0.65 mg.	+2%
8	1.0 c.c.	....	0.01 c.c.	0.1 c.c.	0.1 c.c.	0.55 mg.	−5%
9	....	1.0 c.c.	3.0 c.c.	0.3 c.c.	0.1 c.c.	0.13 mg.	−3%
10	....	1.0 c.c.	1.0 c.c.	0.1 c.c.	0.1 c.c.	0.20 mg.	+7%
11	....	1.0 c.c.	0.1 c.c.	0.1 c.c.	0.1 c.c.	0.24 mg.	+12%
12	....	1.0 c.c.	0.01 c.c.	0.1 c.c.	0.1 c.c.	0.16 mg.	+1%

In this experiment an emulsion of fresh typhoid bacilli, total nitrogen 1.4 milligrams, and fresh staphylococci, total nitrogen 0.715 of a milligram, were used, permitted to remain with typhoid immune serum and complement for sixteen hours at 37° C., and then washed and digested with 0.1 of a cubic centimeter of trypsin solution. In the controls the typhoid digestion was 0.625 of a milligram, staphylococci digestion 0.15 of a milligram. It will be noted that with three cubic centimeters of the immune serum there was 6 per cent. less digestion than in the control; with one cubic centimeter an equal amount; with 0.1 of a cubic centimeter, a slight increase; while with 0.01 of a cubic centimeter, there was a drop to an amount less than the control. This action was not specific, for when staphylococci and the typhoid immune serum were used a similar and even more pronounced result was obtained. We are inclined to attribute this result to the adsorption by the bacteria of lipoids from the serum, which, if sufficient, as when larger amounts of serum are permitted to act, will protect the bacteria from

tryptic digestion, and overcome the alteration produced by the immune body which under ordinary circumstances seems to render the bacterial body less resistant to trypsin.

In order to avoid the presence of an excess of antibody we have sensitized bacteria with immune serum. They were then washed, treated with complement, washed again, and tryptic digestion of the sensitized and unsensitized bacteria was permitted.

PROTOCOL VI.

Effect of Sensitization of Typhoid Bacilli on Their Tryptic Digestibility.

Tube No.	Typhoid bacteria.	Sensitized typhoid bacteria.	Complement.	Trypsin.	Non-coagulable nitrogen in supernatant fluid.	Tryptic digestion.
1	1.0 c.c.		Total nitrogen = 0.55 mg., total non-coagulable nitrogen = 0.05 mg. per c.c.			
2	....	1.0 c.c.	Total nitrogen = 0.575 mg., total non-coagulable nitrogen = 0.00 mg. per c.c.			
3	1.0 c.c.	....	....	0.1 c.c.	....	0.162 mg.
4	....	1.0 c.c.	....	0.1 c.c.	....	0.172 mg.
5	1.0 c.c.	....	1.0 c.c.	....	0.31 mg.	....
6	1.0 c.c.	....	0.1 c.c.	....	0.12 mg.	....
7	....	1.0 c.c.	1.0 c.c.	....	0.35 mg.	....
8	....	1.0 c.c.	0.1 c.c.	....	Lost	....
9	....	....	1.0 c.c.	....	0.305 mg.	....
10	....	....	0.1 c.c.	....	0.06 mg.	....
11	1.0 c.c.	....	1.0 c.c.	0.1 c.c.	....	0.172 mg.
12	1.0 c.c.	....	0.1 c.c.	0.1 c.c.	....	0.15 mg.
13	....	1.0 c.c.	1.0 c.c.	0.1 c.c.	....	0.316 mg.
14	....	1.0 c.c.	0.1 c.c.	0.1 c.c.	....	0.25 mg.

In protocol VI there was no increase in autolysis of the two series of bacteria. This will be seen from tubes 5 to 8, the corresponding serum controls being tubes 9 and 10. The sensitized bacteria are much more easily digested by trypsin (tubes 13 and 14) than the unsensitized bacteria (tubes 11 and 12).

DISCUSSION.

In view of the established fact that the serum of practically all animals contains a markedly active antiferment against trypsin, leucoprotease, and the autolytic ferments,—a non-specific substance which in a neutral or slightly alkaline reaction inhibits protease

action of any kind and is essential to life by preventing the splitting of native proteins in the serum to toxic products,—we can hardly expect a result other than that noted in the above experiments; *i. e.*, the fact that bacteriolysis is not associated with a proteolysis. Jobling and Strouse (7) have called attention to the fact that the mere solution of bacteria should not be confused with proteolysis; the former might be due to purely physical factors, as when organisms are kept under lipoid solvents as preservatives. This distinction should be made with equal emphasis for the immunity reactions.

If bacteriolysis is not associated with proteolysis we must find some other explanation. This, we believe, should be sought in the relation of the lipoids of the bacteria and the lipolytic effect of the serum (Jobling and Bull (8)). That a physical change of some sort is induced in corpuscles treated with immune serum and complement has been shown by Eisner and Friedemann (9), and is indicated by the fact that bacterial organisms are rendered more digestible for trypsin. This might be due to (a) an alteration in the lipoidal surface membranes, rendering the organism more permeable for trypsin; (b) an oxidation, rendering the antiferments of the bacteria less active; or (c) the death of the organism (expressing, of course, a physical change) with resulting alteration of the balance of the bacterial protein and lipoids and consequent loss of antitryptic activity.

Our results might be considered to contradict directly the results obtained by several workers with the Abderhalden dialysis method, who have observed an increase in protein split products when bacteria were allowed to digest with immune serum (Voelkel (10)). These workers have ignored the presence of an excess of antiferment in the blood, assuming that proteolysis might take place under normal conditions with resulting splitting of bacteria. Inasmuch as Plaut (11) has recently shown that such absorbing substances as kaolin, starch, etc., give an equally positive reaction, and since de Waele (12) has just demonstrated the same fact by the simple means of precipitating the globulins of the serum, there can be no question that the idea of a specific protease action is unfounded. This view has also been taken by Kirschbaum and Köhler (13), who have been unable to obtain results by means of the dialysis method which

could in any way be interpreted as indicating a specific protease action upon bacteria. This, of course, does not hold true for the peptases, there being no antiferments in the serum against these ferments. Reasoning from a different line of experiments, based on the demonstration of the lipoidal nature of the antiferments (14), we have shown that serum from which the antiferment has been removed will autolyze (15) and that the antiferment can be removed by adsorption (16), after which there is, of course, no further impediment to either protease or peptase action. It is, therefore, more than probable that the dialyzable split products obtained by various workers from the bacterial digestion by immune serum represent digestion products brought about by an alteration in the balance of the ferment and antiferment when the latter is adsorbed by the bacteria. It is more easy to conceive that the soluble serum proteins are split when their antiferment is adsorbed than that the bacterial protein, protected by its lipoids and membranes, should be attacked.

CONCLUSIONS.

1. There is no demonstrable increase in non-coagulable nitrogen during bacteriolysis.

2. Bacteria treated with immune serum and complement are so altered that they are more readily digested by trypsin.

3. Bacteria treated with complement alone become more resistant to proteolysis.

4. Bacteria treated with an excess of immune serum and complement become more resistant to proteolysis.

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STUDIES ON THE CHEMISTRY OF SEROUS EFFUSIONS.*

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The formation of serous effusions in different diseases and particularly in nephritis has been the subject of much study and discussion. Although the results of analyses of serous fluids recorded in the literature show striking variations in the composition of such fluids in different pathological conditions, they throw but little light on the chemical factors concerned in their formation. This failure to account for the differences in the chemical composition of such fluids is due to two causes: (1) a lack of definite knowledge of the processes by which serous fluids of different chemical composition are formed normally in different parts of the body, and (2) the fact that there are relatively few studies on the subject in which simultaneous examinations of blood serum and serous fluids were made.

Recent studies by the author on the chemical composition of blood serum show that certain changes occur in different diseases.[1] Particularly striking is the change from normal in the composition of the protein colloids and other constituents which takes place in parenchymatous nephritis, in which effusions are of common occurrence, as contrasted with other forms of renal disease, in which effusions do not occur.[2]

Without attempting to enter into a discussion at the present time of the relation of the chemical changes in the blood serum to the nature and occurrence of effusions in disease, I wish to put on record the chemical findings of forty-three puncture fluids, some of which were made simultaneously with analysis of blood serum.

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[1] Epstein, A. A., *Jour. Exper. Med.*, 1912, xvi, 719.

[2] Epstein, *idem*, 1913, xvii, 444.

The analyses of the effusions were carried out along lines similar to those followed in the study of the blood sera.[3] The substances determined were the proteins, chlorides, and incoagulable and non-protein nitrogen. Ash and total solids were also estimated in several of the fluids. A number of the fluids which were opalescent and of a chyliform appearance were analyzed for fats and other ether-soluble substances. These, however, will not be reported in the present study.

The fluids subjected to analysis may be grouped according to the following scheme which is based on clinical data.

1. Cutaneous effusions: (*a*) nephritic and (*b*) cardionephritic.
2. Pleural effusions: (*a*) inflammatory and (*b*) non-inflammatory including (1) nephritic, (2) cardiac, (3) cardionephritic, and (4) new growth.
3. Abdominal effusions: (*a*) inflammatory, tuberculous, and (*b*) non-inflammatory, including (1) new growth, (2) cirrhosis of the liver, (3) cardiac, and (4) cardionephritic.
4. Hydrocele fluid.

CUTANEOUS EFFUSIONS.

TABLE I.

Cutaneous Effusions.

Case No.	Total protein.	Incoagulable nitrogen.	Total globulin.	Euglobulin.	Pseudoglobulin.	Albumin.	Chlorides.	Total solids.	Ash.	Ratio of globulin to albumin.	Per cent. of globulin in protein.
	Gm. per 100 c.c. of fluid.										
					Nephritis.						
4	0.098	0.004	0.080	0.048	0.032	0.018	0.406	1.230	0.980	1:0.24	81
44	0.171	0.162	0.104	0.091	0.095	......	0.397	......	......	1:0.60	56.1
29	0.145	0.063	0.079	0.024	0.055	0.066	0.433	1.357	0.870	1:0.84	54
					Cardionephritis.						
14	0.462	0.035	0.155	0.032	0.123	0.307	0.412	1.515	0.950	1:2.5	28.5
70	0.119	0.035	0.043	0.018	0.025	0.076	0.420	......	......	......	30
201	0.100	0.053	0.018	......	......	0.082	0.400	......	......	......	18

The six cutaneous fluids (table I), of which three are of nephritic and three of cardionephritic origin, have certain features in com-

[3] Epstein, *Jour. Exper. Med.*, 1912, xvi, 719.

mon and differ in certain others. The protein content of all these fluids is very low, ranging in the nephritic group from 0.098 of a gram to 0.171 of a gram per 100 cubic centimeters, and in the other group from 0.100 of a gram to 0.462 of a gram per 100 cubic centimeters of fluid. The two classes of fluids differ in the percentage relations of albumin and globulins. Of the three nephritic fluids the globulins constitute 81 per cent., 56.1 per cent., and 54 per cent., respectively, of the total protein. The proportion of globulins in the second group of fluids is much lower; namely, 28.5 per cent., 30 per cent., and 18 per cent., respectively.

Although the amount of protein in these fluids is far below that of blood sera, the percentage of globulin in each group of cutaneous fluids is similar to that of the corresponding group of blood sera. Fluids 4 and 201 are from cases whose blood serum was examined at the same time. Table II shows the values obtained in the analysis of the corresponding serum and edema fluids.

TABLE II.

Case 4. Nephritis.

		Edema fluid.	Blood serum.
Gm. per 100 c.c. of fluid	Total protein	0.098	5.125
	Incoagulable nitrogen	0.040	0.280
	Total globulin	0.080	4.325
	Albumin	0.018	0.800
	Chlorides	0.406	0.412
	Total solids	1.230	.....
	Ash	0.980	.....
	Per cent. of globulin	81.0	83.0

Case 201. Cardionephritis.

		Edema fluid.	Blood serum.
Gm. per 100 c.c. of fluid	Total protein	0.100	3.330
	Incoagulable nitrogen	0.053	0.154
	Chlorides	0.400	0.435
	Globulin	0.018	2.381
	Albumin	0.082	0.949
	Per cent. of globulin	18.0	71.5

In both cases the quantity of protein in the effusions is very small as compared with that of the blood serum. In the nephritic effusion the percentile relation of the protein fractions corresponds to that of the blood serum. In the cardionephritic case this parallelism does not exist.

The chloride content of these fluids is uniformly high when compared with that of normal blood sera, but much lower than the content in the blood sera of corresponding cases (compare fluids 4 and 201 and their corresponding blood sera). The total solids are low in all the fluids analyzed. The ash, on the other hand, is practically the same as that found in normal blood sera. The incoagulable and non-protein nitrogen in all but one fluid (44) is very low when compared with the blood sera belonging to these cases. In this respect the incoagulable nitrogen is like the chlorides, except that the difference in the quantity of incoagulable nitrogen in the effusions and in the blood sera is much greater than that exhibited by the chlorides.

A comparison of the results obtained in the analyses of the subcutaneous fluids with those of other effusions shows that their composition is unique. They are of a pale watery appearance like lymph, containing very little protein (less than 0.5 per cent.) and a large amount of chlorides. The incoagulable nitrogen is relatively low, certainly far below that found in the blood sera of the cases examined.

PLEURAL FLUIDS.

Most of the pleural fluids analyzed (table III) are of inflammatory origin. Fluids 20, 21, 54, 60, 61, 80, and 204 are from cases of pleurisy of unknown etiology. Fluids 52, 53, and 206 are from cases of probable tuberculosis, and fluid 30 is from a case of empyema. The remaining effusions are presumably of non-inflammatory origin; two (205 and 56) are from cases of nephritis; of the others, 63 is from a case of intrathoracic lymph sarcoma, and 202 and 203 are also due to neoplasms of undetermined nature.

All of the above fluids are rich in protein, the amounts ranging from 1.250 grams to 6.250 grams per 100 cubic centimeters. The inflammatory fluids of unknown etiology (20, 21, 54, 60, 61, 80, and 204) show a moderate elevation of the percentage of globulin as compared with the content of globulin in normal blood serum. The incoagulable and non-protein nitrogen found in these fluids present values which are for the most part somewhat above the standard figure for blood serum. These values vary from 0.049 to 0.080 of a gram per 100 cubic centimeters of fluid. Likewise the chloride content of these fluids is higher than that of normal blood serum.

TABLE III.

Pleural Effusions.

Case No.	Total protein.	Incoagulable nitrogen.	Total globulin.	Euglobulin.	Pseudoglobulin.	Albumin.	Chlorides.	Ash.	Ratio of globulin to albumin.	Per cent. of globulin in protein.
	Gm. per 100 c.c. of fluid.									
	Inflammatory. Unknown etiology.									
20	4.400	0.046	1.780	0.470	1.310	2.620	0.362	0.650	1:1.5	40
21	3.969	0.080	1.882	0.392	1.490	2.087	0.355	0.650	1:1.1	47.6
60	3.831	0.050	1.437	0.650	0.787	2.394	0.404	......	1:1.6	39.3
61	3.412	0.045	2.537	0.569	1.968	0.875	0.412	......	1:0.34	74.3
54	4.781	0.067	2.056	0.881	1.175	2.715	0.397	......	1:1.3	43
80	5.487	0.049	2.625	0.822	1.803	2.862	0.412	......	1:1.1	47.6
204	6.775	0.022	2.187	4.588	......	......	0.391	......	1:2.14	32
	Inflammatory. Tuberculosis.									
52	4.275	0.022	2.387	1.181	1.206	1.888	0.372	......	1:0.8	56
53	6.250	0.056	2.969	0.812	2.157	3.291	0.376	......	1:1.1	47.6
206	5.231	0.039	3.506	......	......	1.725	0.405	......	1:0.48	67
	Inflammatory. Empyema.									
30	5.019	0.086	1.027	0.625	0.402	3.992	0.372	......	1:3.8	20.8
	Non-Inflammatory. Nephritis.									
56	3.581	0.060	1.469	0.631	0.838	2.112	0.426	......	1:1.4	41
205	1.250	0.164	0.875	......	......	0.375	0.426	......	1:0.3	70
	Non-Inflammatory. Neoplasms.									
63	4.250	0.053	1.776	0.490	1.280	2.484	0.390	......	1:1.4	41.8
202	5.023	0.038	......	......	......	......	0.440	......	....	....
203	4.950	0.048	......	......	......	......	0.433	......	....	....

Four fluids in this series are of special interest (20, 21, 60, and 61). They represent fluids which were obtained from two patients upon whom the paracentesis was repeated. Fluids 20 and 21 were removed from the chest of a patient at an interval of three days. Notwithstanding the short interval the two fluids show considerable differences in composition. The fluid from the second paracentesis (21) contains less protein than the first,—3.969 grams as compared with 4.400 grams per 100 cubic centimeters; but the ratio of globulin to albumin is higher by 7.6 per cent. than that present in the fluid from the first paracentesis. The incoagulable nitrogen in the second fluid is almost double that of the first. The chloride content, on the other hand, is slightly lower. The ash content is the same in

both fluids, and if we compute the chlorides in these fluids as sodium chloride, then we obtain values of 0.613 and 0.592 of a gram per 100 cubic centimeters, respectively, which leaves a balance of 0.037 of a gram and 0.058 of a gram per 100 cubic centimeters of fluid for the other salts in the ash.

The second set of effusions show different changes in composition from those observed in the fluids just described. In this instance the second paracentesis was performed twelve days after the first. The fluid from the second paracentesis (61) has a lower protein content than the first (60), *i. e.*, 3.412 grams as compared with 3.831 grams per 100 cubic centimeters. The incoagulable nitrogen falls slightly, whereas the chloride content rises considerably. The most striking change occurs in the percentage of globulin; it rises from 39.3 per cent. to 74.3 per cent. In both sets of analyses it is found that, whereas the percentage of total globulin rises in the second fluid, the percentage of euglobulin to pseudoglobulin falls. This is especially marked in the second set of fluids (60 and 61). It is likely that the cause for the change in the quantitative relations of the globulin rests on a difference in the rate of secretion or resorption of the albumin and globulin fractions of the serum. This, however, will be considered at another time.

The three fluids from the tuberculous cases are in most respects like the other effusions of inflammatory origin. They possess a high protein content. One of these (53) contains an unusually large amount of protein, practically as much as blood serum. It may be added that in this particular fluid a very unusual amount of fibrin was found.

Fluid 30 is from a case of empyema, resulting from a pneumonic infection. This fluid possesses a high protein content, a large amount of incoagulable nitrogen, a small amount of chlorides, and a very low percentage of globulin (20.8 per cent.). In this latter fact we find a contradiction to the usual statement and belief that the serum of purulent fluids is rich in globulins, because of the presence of disintegrating pus cells in the fluid.

In its general character the fluid from a case of intrathoracic lymphosarcoma resembles the fluids that are of inflammatory origin. It does not present any distinguishing features.

The last fluids in this series are from cases of chronic nephritis. In their protein content these are the lowest in the series. The incoagulable nitrogen in one of these (56) is only moderately elevated, whereas the chlorides are rather high. In the second fluid both groups of substances show very high values.

The chemical relation of these fluids to the blood sera of the corresponding cases can be seen in table IV.

TABLE IV.

Case 56.

		Pleural fluid.	Blood serum.
Gm. per 100 c.c. of fluid	Total protein	3.581	7.525
	Incoagulable nitrogen	0.060	0.120
	Total globulin	1.469	2.537
	Albumin	2.112	4.988
	Chlorides	0.426	0.480
	Per cent. of globulin	41.7	34.0

Case 205.

		Pleural fluid.	Blood serum.
Gm. per 100 c.c. of fluid	Total protein	1.250	7.087
	Incoagulable nitrogen	0.164	0.187
	Globulin	0.875	4.775
	Albumin	0.375	2.312
	Chlorides	0.426	0.412
	Per cent. of globulin	70.0	67.0

As table IV shows, striking differences exist between the composition of the effusion fluids, and that of the corresponding blood sera. In one instance (56) the pleural fluid contains less than half of the protein found in the blood serum. In the other case (205) there is even less protein, constituting less than 18 per cent. of that in the blood serum. The incoagulable nitrogen in the first of these fluids is half of that of the corresponding blood serum, in the second it constitutes almost 90 per cent. The chloride in one fluid (56) is lower than the value found in its related blood serum. In the second fluid the chloride content exceeds that of the blood serum.

The percentage of globulin in each fluid corresponds to that of the blood serum from the same case, but in both it is higher than that of the blood serum.

The differences observed in the composition of the pleural fluids and that of the blood sera from the same cases are not easy to

explain. The difference in the amounts of incoagulable nitrogen and chlorides indicates two points: first, that the diffusion of the organic bodies constituting the incoagulable nitrogen is of a different order from that of the inorganic salts; and second, that the difference in the content of these two groups of substances in the pleural fluid and the blood cannot be accounted for by the accepted rules of diffusion and osmosis alone.

ABDOMINAL FLUIDS.

Of the abdominal effusions twenty fluids were examined (table V). Of these, one is of tuberculous origin, two are due to neoplasms of abdominal viscera, nine are due to cardiac insufficiency, four are produced by cirrhosis of the liver, and four occurred in nephritis. One of the latter was from a case of pure nephritis and the other three were from nephritis complicated by disease of the heart.

The character of these fluids is like that of the pleural fluids of the same origin. The two carcinomatous fluids contain moderate amounts of protein, of which half or more is globulin. The incoagulable nitrogen in these two fluids is comparable with the incoagulable nitrogen content of normal blood serum. The chloride content presents no unusual values. In the tuberculous fluid the protein content is moderate in amount, but very much less than that found in pleural fluids of similar origin. The percentage of globulin is equivalent to that of blood serum. The chloride content is high. The other ingredients also closely approximate the values usually obtained in the examination of the blood.

The abdominal fluids of cardiac origin appear to stand in etiologic relationship to static disturbances. The composition of these fluids, with one or two exceptions, is more or less uniform. Their protein content varies between 1.567 and 4.712 grams per 100 cubic centimeters. In seven of the fluids the protein ranges above 3 grams. In nearly all of the latter the globulin content is the same as that found in the serum of normal blood. The same is true of the incoagulable nitrogen. With but two exceptions (7 and 69), the chloride is considerably elevated, ranging from 0.362 of a gram to

0.454 of a gram per 100 cubic centimeters. In one instance (7) the ash was also determined, and was found to be equal to that of normal blood serum.

TABLE V.

Abdominal Effusions.

Case No.	Total protein.	Incoagulable nitrogen.	Total globulin.	Euglobulin.	Pseudoglobulin.	Albumin.	Chlorides.	Total solids.	Ash.	Ratio of globulin to albumin.	Per cent. of globulin in protein.
	Gm. per 100 c.c. of fluid.										
	Tuberculous and New Growths.										
105	1.725	0.045	0.281	......	......	1.444	0.398	......	......	1:5.1	16.3
209	3.725	0.040	1.838	......	......	1.887	0.385	......	......	1:1.+	49
208	3.081	0.043	1.462	......	......	2.219	0.418	......	......	1:1.5	39.7
	Cardiac.										
7	2.080	0.037	0.882	0.189	0.693	1.198	0.362	3.400	0.958	1:1.36	42.3
28	1.567	0.049	0.692	0.275	0.417	0.975	0.454	......	......	1:0.6	62
40	3.675	0.084	1.919	0.625	1.294	1.756	0.412	......	......	1:0.9	52.2
69	4.694	0.055	1.787	0.750	1.037	2.907	0.376	......	......	1:1.6	38
81	4.712	0.055	1.656	0.456	1.200	3.056	0.404	......	......	1:1.9	34
32	3.230	0.053	1.062	0.372	0.690	2.168	0.412	......	......	1:2	33.3
66	3.702	0.076	1.462	0.500	0.962	2.240	0.418	......	......	1:1.5	39.9
68	3.123	0.077	1.331	0.463	0.868	1.792	0.404	......	......	1:1.3	42.6
207	3.381	0.040	......	......	......	......	0.440	......	......	......	......
	Cirrhosis of the liver.										
15	3.332	0.090	1.625	0.525	1.100	1.707	0.428	4.815	0.950	1:1.05	49
26	3.017	0.051	1.012	0.205	0.707	2.005	0.390	......	......	1:2.0	33.3
67	0.521	0.039	0.325	0.125	0.200	0.193	0.326	......	......	1:0.6	62.3
79	0.686	0.038	0.400	0.131	0.769	0.256	0.497	......	......	1:0.6	62.3
	Nephritis.										
1a	0.285	0.035	0.285	......	......	......	0.420	1.040	0.875	......	100
	Cardionephritis.										
32	3.230	0.053	1.062	0.372	0.690	2.168	0.412	......	......	1:2	33.3
66	3.702	0.076	1.462	0.500	0.962	2.240	0.418	......	......	1:1.5	39.9
68	3.123	0.077	1.331	0.463	0.868	1.792	0.404	......	......	1:1.3	42.6

In one case (table VI) both the blood serum and the ascitic fluid were examined. The protein content of the ascitic fluid is less than half of that of the blood serum, but the percentage of globulin is nearly double that of the other. In other respects the two fluids are alike.

Of the fluids arising in cases of cirrhosis of the liver, two (15 and 26) resemble the abdominal fluids of cardiac origin; the others differ considerably. The first two contain over 3 grams of protein per 100 cubic centimeters of fluid, and the percentage of globulin is the same as that found in the fluids of cardiac origin. The same

TABLE VI.

Case 207.

		Ascitic fluid.	Blood serum.
Gm. per 100 c.c. of fluid	Total protein...........	3.381	7.575
	Incoagulable nitrogen....	0.040	0.040
	Globulin................	1.481	1.956
	Albumin................	1.900	5.619
	Chlorides...............	0.440	0.433
	Per cent. of globulin.....	43.0	26.0

is true of the incoagulable nitrogen and chloride contents. In the other two fluids (67 and 79) the protein content is rather low,—slightly over 0.5 of a gram per 100 cubic centimeters,—but the globulin fraction is very high in both, being 62.3 per cent. The incoagulable nitrogen in these two effusions is low, or comparable to that of normal blood serum. The chloride content in one fluid (67) is very low, 0.326 of a gram per 100 cubic centimeters, in the other exceedingly high, 0.497 of a gram per 100 cubic centimeters. It would have been of interest to compare the quantities of the different ingredients with those of the blood serum of the corresponding cases, but these were not available.

Four abdominal fluids of renal origin were examined. Of these only one is from a case of pure nephritis. The others are from cases of nephritis complicated by heart disease. The latter resemble the composition of the fluids of cardiac origin described above. The protein content is over 3 grams to 100 cubic centimeters. The incoagulable nitrogen is slightly elevated; the chloride content is high, but unlike the other fluids. The globulin percentage is low, averaging 38.6 per cent. In this the fluids resemble normal blood serum. The fluid of purely nephritic origin (1a) contains a small amount of protein (0.285 of a gram to 100 cubic centimeters), but all of it is globulin. In this respect this fluid is different from all the other fluids examined excepting the subcutaneous effusions

of nephritic origin. The incoagulable nitrogen is low, but the chloride content is very high, 0.420 of a gram per 100 cubic centimeters. The value obtained in the analysis of the ash is low and considerably under that of normal blood serum. If we deduct from the quantity of ash the salt equivalent of the chlorides in it, there remains but a small balance for other salts.

The abdominal fluid just described gains in interest when the results are compared with values obtained in the analysis of the blood sera from nephritic cases.[4]

HYDROCELE FLUID.

One hydrocele fluid was also examined; the results are shown in table VII. This fluid is rich in protein, but its globulin content is

TABLE VII.

Hydrocele Fluid.

Case No.	Total protein.	Incoagulable nitrogen.	Total globulin	Euglobulin.	Pseudoglobulin.	Albumin.	Chlorides.	Total solids.	Ash.	Ratio of globulin to albumin.	Per cent. of globulin in protein.
	Gm. per 100 c.c. of fluid.										
17	5.332	0.061	1.100	0.270	0.830	4.232	0.412	6.960	0.945	1:3.8	20.7

low. The chlorides are rather high, as is the ash. The other ingredients present nothing striking.

SUMMARY.

A comparison of the results obtained in the analysis of the different effusions shows that they vary, (1) according to location, and (2) according to the disease in which they are produced. Thus the subcutaneous effusions are totally different in their composition from the abdominal or pleuritic fluids; and again the abdominal fluid of nephritic origin is different from those of cardiac or other origin.

The cutaneous effusions are characterized by a very low protein content and a small amount of incoagulable nitrogen. Of the protein present in these fluids, the globulin constitutes the greater

[4] Epstein, *Jour. Exper. Med.*, 1912, xvi, 719.

portion. In the mixed cardionephritic fluids the ratio of globulin to albumin is lower than that in normal blood serum; but when compared with the blood sera of cardionephritic cases there appears to be a certain parallelism. The chloride content of these fluids is considerably above that found in normal blood serum, but is comparable with those of corresponding cases.

The effusions occurring in serous cavities differ from the cutaneous ones by their higher protein content. The highest values are attained in the pleural fluids, in which the protein present is almost the same as that in normal blood serum. The incoagulable nitrogen in these fluids is uniformly low. The chlorides vary in amount according to the nature of the case. In the effusions of inflammatory origin the values are lower than those found in the blood. In the pleural fluids of other than inflammatory origin the chloride content is either the same or higher than that of blood serum. The globulin-albumin ratio in all of these fluids except one[5] (table III, fluid 61) is higher than that of normal blood serum. Otherwise the highest value is presented by a pleural fluid from a case of nephritis (table III, fluid 205) and the lowest by an empyema fluid (table III, fluid 30). The latter case is of especial interest because of the theory, first propounded by Schmidt, that the leucocytes in the purulent fluid are largely responsible for high globulin content.

The abdominal fluids in general are less rich in protein than the pleural effusions. Those of cardiac origin give the highest protein values, but the globulin-albumin ratios are lower than in the pleural fluids or in the blood sera of corresponding cases. Only in two fluids does the globulin reach a percentage of 62 and 52.2. In both these cases the chloride content is also high. The incoagulable nitrogen in all of them is rather low. In one fluid of purely nephritic origin the protein content is low and all of it is globulin. This is suggestive, when compared with the values obtained in the analysis of the blood sera from nephritic cases. The results obtained in the analysis of abdominal fluids from cardionephritic cases approach very closely those obtained in purely cardiac cases.

[5] This fluid was obtained at a second tapping. The fluid obtained at the first paracentesis is shown in table III, fluid 60.

PNEUMOCOCCUS HEMOTOXIN.*

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It has been generally held that pneumococci or their products do not possess the power of producing lysis of red blood cells. On the other hand, it has long been known that the culture filtrates of certain bacteria, such as staphylococcus, certain races of streptococcus, and, above all, *Bacillus tetani*, possess this property to a marked degree. The properties of the hemolytic toxin produced by the last named organisms have been thoroughly studied by Madsen and others, and through this study many of the facts concerning hemolytic toxins have been discovered. Since such hemolytic toxins may be filtered, and since they may act as antigens, they may be considered true toxins in the Ehrlich sense. Old cultures of other bacteria, such as *Bacillus pyocyaneus* and *Bacillus anthracis*, may also be hemolytic, but the production of true hemolytic toxins by these organisms is considered doubtful. Indeed, very old cultures of practically all bacteria may produce hemolysis, but it is possible that this lytic effect, in certain of the cases at least, is due directly to changes in reaction of the old culture medium.

The property of certain races of streptococci of producing hemolysis has been considered by Schottmüller and others to be of great value in differentiating between the different varieties, and also between pneumococcus and the virulent streptococci, the so called *Streptococcus hemolyticus*. The usual method of determining whether bacteria possess this property is to grow them on agar plates containing blood, when in the case of hemolytic bacteria, such as *Streptococcus hemolyticus*, the colonies become surrounded by a transparent zone contrasting with the opacity of the rest of the medium, while in the case of non-hemolytic bacteria, such as pneumococcus, no such clear zones are seen.

* Received for publication, August 1, 1914.

While the power to produce hemolysis in culture medium is not possessed by pneumococci, or, if so, to a very slight degree, observations which we have made indicate that the bodies of pneumococci contain a substance or substances, which when set free are actively hemolytic, and that the serum of animals immunized to the bacteria or to the bacterial substance has increased power of neutralizing this lytic poison. These lytic substances differ from the so called bacterial hemotoxins in that they are contained within the bacterial cells and are only set free on the dissolution of the latter, but they may, nevertheless, have as great a pathological significance.

The first observations were made when studying the properties of a poison produced by dissolving washed pneumococci in dilute solutions of bile or sodium cholate.[1] The solution so obtained, which produces acute death in guinea pigs on intravenous injection, was also found to be hemolytic when added to an emulsion of red blood corpuscles in salt solution. At first this hemolytic property was thought to be due to the sodium cholate contained in the solution, but careful titration of the hemolytic power of the toxin showed that it was much greater than could be accounted for by the contained sodium cholate. In certain experiments, three hundred times as much sodium cholate in salt solution was required to produce hemolysis of 0.5 c.c. of an emulsion of sheep corpuscles as was contained in a minimal lytic dose of the toxin. It is not likely that the mere presence of bacterial substance increases the activity of cholate solutions, since the addition of protein to a cholate solution lessens its activity, and even peptone was found to have no intensifying action, but a slightly inhibiting action instead.

Later experiments have shown conclusively that cholate plays no important part in the reaction, since the lytic substances are present in extracts of pneumococci, obtained by allowing pneumococci to undergo autolysis in salt solution and also in extracts prepared by freezing and grinding the bacteria, in both cases without adding any cholate whatsoever.

PREPARATION OF THE TOXIN.

In the study of the lytic effect of substances obtained from the bodies of pneumococci, the extracts have been prepared in one of the following ways:

(1) Pneumococci are grown for twenty hours in broth, removed from the broth by centrifugalization, and washed once in 0.85 per

[1] Cole, R., *Jour. Exper. Med.*, 1912, xvi, 644.

cent. salt solution. A test of the hemolytic power of toxins made from cultures grown for various lengths of time has shown that the toxins made from cultures twenty to twenty-four hours old are the most active. Toxins made from forty-eight-hour cultures possess little hemolytic power, while those made from seventy-two-hour cultures have no lytic power whatever. This difference is probably associated with the lysis of the bacteria which goes on in old cultures. The washed bacterial sediment is taken up in a small amount of salt solution, usually ten cubic centimeters for the bacteria from one liter of broth culture, and an amount of a 2 per cent. sodium cholate solution barely sufficient to cause solution of the bacteria is added. Usually one cubic centimeter is sufficient. After lysis has occurred, the solution is diluted with salt solution. If the bacteria were obtained from one liter of culture, the solution is usually made up to one hundred cubic centimeters. Different races of bacteria differ in the readiness with which they dissolve in cholate solution. Moreover, different solutions of bacterial bodies obtained in this way differ in their power to produce death in guinea pigs and also to produce hemolysis. Toxins, as above described, however, usually produce acute death in guinea pigs in doses of three to four cubic centimeters, and hemolysis of 0.5 of a cubic centimeter of sheep corpuscles in doses of 0.02 of a cubic centimeter or less, the whole mixture of toxin and corpuscles being made up to 2.5 cubic centimeters with salt solution.

(2) Pneumococci are grown in broth, washed, frozen, and ground, and the powder is dissolved in salt solution. A diluted toxin so prepared, which kills a guinea pig acutely in doses of three to four cubic centimeters, also usually produces hemolysis of sheep corpuscles, with the technique above described, in doses of 0.02 of a cubic centimeter or less.

Toxins prepared in the above ways are identical in their reactions so far as studied, and in the following pages no mention will be made of the method of preparation in each individual experiment.

A few experiments have also been conducted with extracts prepared by allowing pneumococci to undergo autolysis in salt solution. A series of tubes were prepared, all containing equal quantities of washed pneumococci and equal quantities of salt solution. These

were kept at 37° C., and from time to time a tube was removed and the hemolytic power of the fluid tested. It was found that the hemolytic power of the emulsion rapidly increased and between six and eight hours was at its maximum. There was a very slight fall in hemolytic power up to eighteen hours, but at twenty-four hours it had markedly diminished and was entirely absent after forty-eight hours.

PROPERTIES OF THE TOXIN.

Toxins prepared by solution of pneumococci are lytic for rabbit, sheep, guinea pig, and human red blood corpuscles. Other corpuscles have not been tested. The hemolytic power is greatest for guinea pig corpuscles, less for sheep and human corpuscles, and least for rabbit corpuscles, but the differences are not striking. The rate of hemolysis depends upon the concentration of the toxin. If the concentration be sufficiently great, complete hemolysis may occur within five to ten minutes at 37° C.

Active toxins have been obtained from pneumococci belonging to all of the four immunological groups.[2]

An attempt has been made to discover whether or not any relationship exists between the virulence of organisms employed and the hemolytic power of the extract, and a larger number of races have been studied with this point in mind. Rosenow[3] has stated that the more virulent races of pneumococci autolyze most readily. This is generally true, but there are many exceptions. Also the more virulent races are more soluble in bile, though to this also there are apparent exceptions. When a series of cultures of pneumococci are tested, these two properties do not bear constant positions with reference to the virulence of the organisms. It may also be stated from our study that the most active hemolytic toxins are usually obtained from those races of pneumococci that have been most lately cultivated from the animal body and are most virulent. But here again there are sufficient exceptions to throw some doubt on the validity of the generalization. The attempt to increase the hemolytic power of the toxin produced from a given race by repeated passage through animals, testing the toxin production from time to time, does not

[2] Dochez, A. R., and Gillespie, L. J., *Jour. Am. Med. Assn.*, 1913, lxi, 727.

[3] Rosenow, E. C., *Jour. Infect. Dis.*, 1912, x, 113.

yield results that are uniform and consistent. With a given race the hemolytic power of the toxin varies markedly from time to time, even though in each test the toxin is prepared in exactly the same way. This variation probably depends somewhat on the luxuriance of the growth, and this on many factors,—the exact composition of the medium, the exact temperature of the thermostat, etc. The amount of autolysis that has gone on in the culture before centrifugalization is also of importance. It is therefore impossible to predict with any given race and culture exactly the strength of the toxin that will be obtained. The conditions are quite different from those obtaining in the production of diphtheria toxin with different races of bacilli. Here races vary markedly in their power to produce toxin, but this property seems to be fixed in certain races, and no matter how long they are grown outside the body or under what unusual conditions, the power of producing toxin in large amounts is preserved.

As regards the relationship between the hemolytic power and the toxic power as tested by intravenous injection into guinea pigs, there is a much more constant relationship. Even here the parallelism is not exact, but actively hemolytic solutions have always been found to be toxic and, with a few exceptions, the reverse is true. During the past two years toxins from a large number of cultures have been tested, and from the results obtained the above conclusions are derived.

The same manipulations which affect the hemolytic power of the toxin also affect the toxic power. It is impossible to draw an absolute conclusion from this that the same substance which produces hemolysis is the cause of the fatal effect in guinea pigs, though this is strongly suggested. On the other hand, symptoms and pathological changes in the guinea pigs do not seem to indicate that the animals die from the effects of hemolysis alone. In these animals hematuria frequently occurs and focal hemorrhages are seen post mortem, but there are no indications of a wide-spread hemolysis either when death occurs acutely or only after several hours. It is possible that, as in tetanus toxin, the effects may be due to two substances occurring together.

The effect of heat, acids, etc., on the toxin as tested by its power

to produce hemolysis, corresponds with the previously reported effects of these agents on the poison as tested by injection into guinea pigs.[4] Heating to 55° C. for one half hour in our experience always destroys the hemolytic power of the toxin. Heating for one and one half hours at 45° C. usually has no effect, though in one experiment the hemolytic power was diminished after heating one half hour at 45° C. Rosenow[5] has stated that if the autolysate of pneumococci be plunged into boiling water and boiled for ten minutes, then quickly cooled in ice water, the toxicity for guinea pigs frequently remains. As far as the hemolytic effect is concerned, this statement does not hold good, for boiling for ten minutes and then plunging into ice water completely destroys its activity. The hemolytic power is slowly lost when the toxin is kept for some time on ice, though the change does not begin until after eighteen to twenty-four hours.

The hemolytic effect of the solution is markedly diminished or entirely lost after passing through a Berkefeld filter. However, even where controls have shown that the filters entirely prevent the passage of bacteria, the filtrate may still possess some hemolytic power, but it is diminished.

Digestion of the hemolytic toxin with trypsin destroys its activity within forty-five minutes. This is shown by the following experiment.

Fairchild's trypsin solution was employed, each cubic centimeter of which contains 300 units. Even dilute solutions of sodium hydroxide may cause hemolysis, so in making these tests a 5 per cent. solution of sodium carbonate was used to render the mixture alkaline.

The following mixtures were prepared, using toxin prepared from frozen and ground bacteria:

(*a*) 12 c.c. toxin.
3 c.c. 0.85 per cent. sodium chloride solution.

(*b*) 12 c.c. toxin.
1.5 c.c. trypsin solution.
1.5 c.c. 0.5 per cent. solution sodium carbonate.

Two series of tests were made (table I). In series 1 the mixtures were made up cold and the sheep corpuscles were added at once. In series 2 the mixtures were incubated at 37° C. for 45 minutes before the corpuscles were added.

[4] Cole, R., *Jour. Exper. Med.*, 1912, xvi, 644.

[5] Rosenow, E. C., *Jour. Infect. Dis.*, 1912, xi, 235.

TABLE I.[6]

Series 1.

Tube No.	1 c.c. of mixture in dilution.	Sodium chloride solution.	Emulsion of sheep corpuscles.	Results. Readings after 3 hrs. at 37° C.	
				Mixture (*a*).	Mixture (*b*).
1	1 : 2	1 c.c.	0.5 c.c.	+	+
2	1 : 4	1 c.c.	0.5 c.c.	+	+
3	1 : 8	1 c.c.	0.5 c.c.	+	+
4	1 : 16	1 c.c.	0.5 c.c.	+	+
5	1 : 32	1 c.c.	0.5 c.c.	+	+
6	1 : 64	1 c.c.	0.5 c.c.	+	+
7	1 : 128	1 c.c.	0.5 c.c.	±	±
8	1 : 256	1 c.c.	0.5 c.c.	o	o
9	1 : 512	1 c.c.	0.5 c.c.	o	o

Series 2.

Tube No.	1 c.c. of mixture in dilution.	Sodium chloride solution.		Emulsion of sheep corpuscles.	Mixture (*a*).	Mixture (*b*).
1	1 : 2	1 c.c.	At 37° C. for 45 min.	0.5 c.c.	+	o
2	1 : 4	1 c.c.		0.5 c.c.	+	o
3	1 : 8	1 c.c.		0.5 c.c.	+	o
4	1 : 16	1 c.c.		0.5 c.c.	+	o
5	1 : 32	1 c.c.		0.5 c.c.	+	o
6	1 : 64	1 c.c.		0.5 c.c.	+	o
7	1 : 128	1 c.c.		0.5 c.c.	+	o
8	1 : 256	1 c.c.		0.5 c.c.	±	o
9	1 : 512	1 c.c.		0.5 c.c.	±	o

Controls made with toxin plus sodium carbonate and with sodium carbonate alone showed that the latter had no effect on hemolysis.

The fact that the action of trypsin destroys the hemolytic effect of the solution obviously does not prove that the toxin is of protein nature. It indicates, however, that the toxin is probably closely associated with the protein constituents.

Attempts to extract the toxic substance with ether have so far proved unavailing. The toxin has been extracted with large amounts of ether, the ether evaporated under a fan in the cold, and the oily residue taken up in a small amount of alcohol and made into an emulsion in salt solution. Such an emulsion, however, has not been found hemolytic. There is no evidence, therefore, that the hemolytic effects are due to fatty or lipoidal constituents of the bacterial cells.

The presence of blood serum is known to inhibit the action of certain hemolytic toxins, not only bacterial toxins but others as well. Experiments with normal serum showed that the action of the pneumococcus hemolytic toxin is also inhibited to some extent by the

[6] In the tables + indicates complete hemolysis; ± indicates partial hemolysis; ϕ indicates a trace of hemolysis; o indicates no hemolysis.

presence of normal horse serum, to a somewhat greater extent by normal sheep and normal human serum, and to a still greater extent by normal rabbit serum. This antihemolytic effect is also possessed by dilute solutions of egg albumen. The effect of mucus contained in the sputum of a patient suffering from pneumonia was also tested. The mucus was shaken in salt solution; this mixture was added to the toxin and after one half hour at 37° C., sheep corpuscles were added. It was found that the mucus also had marked inhibiting power. Noguchi[7] has brought evidence to show that the inhibiting effect of normal blood serum for tetanolysin is due, in part at least, to the presence of cholesterin. It was therefore important to determine whether or not the hemolytic effect of the pneumococcus toxin was inhibited by the presence of cholesterin.

A protocol of one of the experiments to determine the effect of cholesterin in inhibiting hemolysis is given below.

Toxin.—June 17, 1914. Toxin was prepared by adding the washed bacteria from 500 c.c. of a 20-hour broth culture of pneumococcus A69 to 2.5 c.c. of salt solution plus 1 c.c. of a 2 per cent. solution of sodium cholate, placing the mixture at 37° C. for 15 minutes and then adding salt solution to 50 c.c.

Cholesterin Emulsion.—Cholesterin crystals were dissolved in a small amount of warm ether, and then sufficient warm sodium chloride solution was added, shaking constantly, to obtain a 1 per cent. emulsion. This was heated over a steam bath for 30 minutes to drive off the ether and filtered.

The experiments (table II) have shown conclusively that exceedingly small amounts of cholesterin are able to inhibit the action of the toxin. It is probable that the inhibiting effect of serum is also due to its cholesterin content. It is unnecessary to discuss the theoretical aspects of this phenomenon here, since the problem has been thoroughly considered by Noguchi and others in connection with the inhibition of tetanus hemolysis by serum. As they have concluded, this inhibition by cholesterin probably indicates that the lipoidal constituent of the red blood cell plays an important part in hemolysis.

It also seemed of importance to determine the effect of lecithin on the hemolytic action of this toxin. Kyes[8] has shown that the presence of lecithin increases the hemolytic action of cobra venom,

[7] Noguchi, H., *Univ. Penn. Med. Bull.*, 1902, xv, 327.

[8] Kyes, P., *Berl. klin. Wchnschr.*, 1902, xxxix, 918.

TABLE II.

Hemolytic Test with Toxin.

No.	Experiments.	Hemolysis. 1 hr. at 37° C.	Hemolysis. 24 hrs. on ice.
1	1 c.c. toxin undiluted + 1 c.c. sodium chloride solution + 0.5 c.c. sheep corpuscles	+	+
2	1 c.c. toxin diluted (1 : 2) + 1 c.c. sodium chloride solution + 0.5 c.c. sheep corpuscles	+	+
3	1 c.c. toxin diluted (1 : 4) + 1 c.c. sodium chloride solution + 0.5 c.c. sheep corpuscles	+	+
4	1 c.c. toxin diluted (1 : 8) + 1 c.c. sodium chloride solution + 0.5 c.c. sheep corpuscles	+	+
5	1 c.c. toxin diluted (1 : 16) + 1 c.c. sodium chloride solution + 0.5 c.c. sheep corpuscles	+	+
6	1 c.c. toxin diluted (1 : 32) + 1 c.c. sodium chloride solution + 0.5 c.c. sheep corpuscles	+	+
7	1 c.c. toxin diluted (1 : 64) + 1 c.c. sodium chloride solution + 0.5 c.c. sheep corpuscles	+	+
8	1 c.c. toxin diluted (1 : 128) + 1 c.c. sodium chloride solution + 0.5 c.c. sheep corpuscles	±	±

Test of Inhibition with Cholesterin.

No.	Experiments.		1 hr. at 37° C.	24 hrs. on ice.
1	1 c.c. toxin diluted (1 : 25) + 1 c.c. cholesterin emulsion (1 : 100)	30 min. at 37° C. 0.5 c.c. sheep corpuscles added to each tube.	o	o
2	1 c.c. toxin diluted (1 : 25) + 1 c.c. cholesterin emulsion (1 : 500)		o	o
3	1 c.c. toxin diluted (1 : 25) + 1 c.c. cholesterin emulsion (1 : 1,000)		o	o
4	1 c.c. toxin diluted (1 : 25) + 1 c.c. cholesterin emulsion (1 : 5,000)		o	o
5	1 c.c. toxin diluted (1 : 25) + 1 c.c. cholesterin emulsion (1 : 10,000)		o	o
6	1 c.c. toxin diluted (1 : 25) + 1 c.c. cholesterin emulsion (1 : 50,000)		ø	±
7	1 c.c. toxin diluted (1 : 25) + 1 c.c. cholesterin emulsion (1 : 100,000)		±	±
8	1 c.c. toxin diluted (1 : 25) + 1 c.c. sodium chloride solution		+	+
1	1 c.c. toxin diluted (1 : 25) + 1 c.c. cholesterin emulsion (1 : 100)	5 min. at 37° C. 0.5 c.c. sheep corpuscles added to each tube.	o	o
2	1 c.c. toxin diluted (1 : 25) + 1 c.c. cholesterin emulsion (1 : 500)		o	o
3	1 c.c. toxin diluted (1 : 25) + 1 c.c. cholesterin emulsion (1 : 1,000)		o	o
4	1 c.c. toxin diluted (1 : 25) + 1 c.c. cholesterin emulsion (1 : 5,000)		o	o
5	1 c.c. toxin diluted (1 : 25) + 1 c.c. cholesterin emulsion (1 : 10,000)		o	ø
6	1 c.c. toxin diluted (1 : 25) + 1 c.c. cholesterin emulsion (1 : 50,000)		ø	±
7	1 c.c. toxin diluted (1 : 25) + 1 c.c. cholesterin emulsion (1 : 100,000)		+	+
8	1 c.c. toxin diluted (1 : 25) + 1 c.c. sodium chloride solution		+	+

the lecithin acting as an activator. He[9] has also been able to form a combination of the active constituent of cobra venom with lecithin. On the other hand, it has been shown that with other hemolytic toxins lecithin may have an inhibiting action.

The following protocols of experiments indicate the effect of lecithin on the pneumococcus hemolytic toxin (table III).

TABLE III.

Tube No.	Experiments of July 2, 1914.		Hemolysis.
1	1 c.c. toxin A69 diluted (1 : 32) + 1 c.c. 1% lecithin emulsion diluted (1 : 10)	30 min. at 37° C. 0.5 c.c. emulsion of sheep corpuscles added to each tube.	0
2	1 c.c. toxin A69 diluted (1 : 32) + 1 c.c. 1% lecithin emulsion diluted (1 : 50)		0
3	1 c.c. toxin A69 diluted (1 : 32) + 1 c.c. 1% lecithin emulsion diluted (1 : 100)		0
4	1 c.c. toxin A69 diluted (1 : 32) + 1 c.c. 1% lecithin emulsion diluted (1 : 500)		+
5	1 c.c. toxin A69 diluted (1 : 32) + 1 c.c. sodium chloride solution		+
6	1 c.c. toxin A69 diluted (1 : 64) + 1 c.c. sodium chloride solution		+
7	1 c.c. toxin A69 diluted (1 : 128) + 1 c.c. sodium chloride solution		+
8	1 c.c. toxin A69 diluted (1 : 256) + 1 c.c. sodium chloride solution		±

The tubes were kept for 1 hr. at 37° C. and 24 hrs. on ice.

The lecithin emulsion was prepared by making a 1 per cent. solution of Merck's lecithin in methyl alcohol, and of this a 10 per cent. emulsion was made in 0.85 per cent. salt solution.

This and other similar experiments have shown that lecithin in low dilutions has slight inhibiting action on the hemolytic effect of the toxin.

To learn whether non-hemolytic doses were rendered hemolytic by the presence of lecithin, experiments like the following were made (table IV).

From this and a number of similar experiments, it is evident that lecithin in no case increases the action of the hemolytic toxin. Except for the slight inhibiting action previously noted, therefore, lecithin has no effect on the hemolytic action of the toxin.

[9] *Kyes, P., Berl. klin. Wchnschr.,* 1903, xl, 956, 982.

TABLE IV.

Tube No.	Experiments of March 31, 1914.	Hemolysis.
1	1 c.c. toxin (1 : 10) + 1 c.c. sodium chloride solution + 0.5 c.c. sheep corpuscles	+
2	1 c.c. toxin (1 : 50) + 1 c.c. sodium chloride solution + 0.5 c.c. sheep corpuscles	+
3	1 c.c. toxin (1 : 100) + 1 c.c. sodium chloride solution + 0.5 c.c. sheep corpuscles	±
4	1 c.c. toxin (1 : 500) + 1 c.c. sodium chloride solution + 0.5 c.c. sheep corpuscles	0
5	1 c.c. toxin (1 : 500) + 1 c.c. lecithin emulsion (1 : 10) + 0.5 c.c. sheep corpuscles	0
6	1 c.c. toxin (1 : 1,000) + 1 c.c. lecithin emulsion (1: 10) + 0.5 c.c. sheep corpuscles	0
7	1 c.c. sodium chloride + 1 c.c. lecithin emulsion (1 : 10) + 0.5 c.c. sheep corpuscles	0

The tubes were kept for 2 hrs. at 37° C.

NATURE OF THE HEMOLYTIC TOXIN.

It is not the purpose of the present paper to discuss the chemical nature of the toxin or its mode of action. Sufficient study of it has not been made to render such a discussion profitable. From the properties already described it is evident that it is closely related to certain other vegetable and animal hemolytic toxins. It is extremely labile, is readily absorbed (as shown by the difficulty with which it passes through a bacterial filter), is destroyed by the action of trypsin, and its action is prevented by the presence of minute amounts of cholesterin and of larger amounts of lecithin.

To the view that it does not exist preformed in the bacterial cell, but is a product arising during self digestion or autolysis, the conclusive objection may be raised that it is present in the solutions prepared by freezing, drying, and grinding the bacteria. During this process there has been no opportunity for autolysis and the conclusion seems justified that the hemolytic substance is contained within the living bacterial cell.

ANTIHEMOLYTIC SERA.

In order to demonstrate that the hemolytic substance under discussion is of the nature of a true toxin, it is necessary to show that it possesses antigenic properties; that is, that its action is inhibited by the serum of animals immunized to it. For purposes of immuniza-

tion rabbits and sheep were employed. These were injected intravenously with increasing doses of the toxin every seven to eight days. The toxins for injection were prepared by dissolving the pneumococci in sodium cholate solution, according to the method previously described, and they were centrifugalized before injection. It is probable that with each injection a few living organisms were also introduced. Since the serum of these animals acquired no agglutinating power for the homologous organisms, however, it is not likely that the acquired properties of the serum were due to the antigenic properties of these few bacteria.

(*a*) *Immune Rabbit Serum.*—The protocol of one experiment is given below (table V).

Rabbit 82–E.—Immunization commenced June 14, 1912. Received 5 doses of toxic extract intravenously during a period of 4 months. The animal received no further injections until June 5, 1913. It then received 7 doses of toxin intravenously at intervals of 6 to 7 days, the last injection being made on July 19. Bled on July 31.

Rabbit 285–A.—Immunization commenced June 12, 1913. Received 7 increasing doses of toxin intravenously, the last one being given on July 19. Animal bled on July 31.

TABLE V.

Sera Tested July 31, 1912. Toxin Prepared from Homologous Organisms.

Experiments.		Hemolysis.			
		Serum 82–E.	Serum 285–A.	Normal rabbit serum.	No serum.
Toxin diluted (1 : 8) + serum diluted (1 : 10)	½ hr. at 37° C.	0	0	±	
Toxin diluted (1 : 8) + serum diluted (1 : 50)		0	0	+	
Toxin diluted (1 : 8) + serum diluted (1 : 100)		0	0	+	
Toxin diluted (1 : 8) + serum diluted (1 : 500)		±	±	+	
Toxin diluted (1 : 8) + serum diluted (1 : 1,000)		±	+	+	
Toxin diluted (1 : 8) + sodium chloride					+

The experiments have been repeated many times with the sera of six rabbits immunized to this toxin. While in no case has the antihemolytic power of the immune serum been greater than 0.002 of a cubic centimeter to two hemolytic units of toxin, in all experiments the antihemolytic power of the immune serum has been considerably

greater than that of normal rabbit serum. Tests of the rabbit sera for agglutinins were made from time to time, but none of the sera acquired the power of agglutinating the homologous organisms.

(*b*) *Immune Sheep Serum.*—Three sheep have been given increasing doses of extracts of pneumococci. Two of these animals were treated with extracts of pneumococci of type I and one received injections of pneumococci of type III, the so called *Pneumococcus mucosus.* The results obtained from the study of the sera of these sheep were identical, and data concerning the serum of but one are given.

Sheep A.—Immunization was commenced May 29, 1913. The toxins for injection were prepared by dissolving pneumococci in sodium cholate solution, as previously described, and the injections were all made intravenously. During the period of about 6½ months the sheep received 18 injections of the toxin. The first injection of the toxin was prepared from the bacteria contained in 12.5 c.c. of a twenty-four-hour bouillon culture. The injections were gradually increased in size. On Dec. 2, 1913, the toxin injected was prepared from the bacteria contained in 1,900 c.c. of a twenty-four-hour bouillon culture. On Dec. 10, an injection was made of toxin prepared from the bacteria contained in 3,000 c.c. of a twenty-four-hour bouillon culture. This injection was apparently too large and probably contained a considerable number of living organisms. Following this injection the animal appeared sick, the temperature was elevated, and the respirations were rapid. After a few days' illness the animal appeared better, but a cough persisted for 2 months with gradual loss of weight and strength, and it died Feb. 10, 1914.

Autopsy.—The pleural and pericardial sacs showed extensive fibrous adhesions. The lungs were edematous. Smears and cultures from the heart's blood showed the presence of pneumococci and a gas-forming anaerobic bacillus morphologically like *Bacillus tetani.* Unfortunately, through an error the cultures were destroyed before the identity of the latter organism could be accurately determined.

TESTS OF SERUM OF SHEEP A.

Agglutination.—The serum was repeatedly tested for agglutination with the homologous organism, the last test being made with serum obtained on December 2, 1913. At no time did the serum possess any agglutinating power.

Protection.—Repeated tests of the protective power of the immune sheep serum for mice were made, employing the technique used in this laboratory for determining protective power.[10] It was found that the serum possessed fairly well marked protective power against pneumococci of type I, and some protective power against

[10] Dochez, A. R., *Jour. Exper. Med.,* 1912, xvi, 665.

pneumococci of other types, except type III, against which no protective power is ever present. The protective power, however, was never so high as that of the serum of horses immunized by injection of living organisms, nor was it so specific.

TABLE VI.

Sheep Serum A, Obtained September 14, 1913. Inactivated. Tested September 15, 1913. Toxin 1.70.

Tube No.	Experiments.		Hemolysis. Normal sheep serum.	Hemolysis. Immune sheep serum A
1	1 c.c. toxin 1.70 + 1 c.c. serum diluted (1 : 10) . . .	½ hr. at 37° C. 0.5 c.c. emulsion sheep corpuscles added to each tube.	0	0
2	1 c.c. toxin 1.70 + 1 c.c. serum diluted (1 : 50) . . .		0	0
3	1 c.c. toxin 1.70 + 1 c.c. serum diluted (1 : 100) . .		0	0
4	1 c.c. toxin 1.70 + 1 c.c. serum diluted (1 : 500) . .		±	0
5	1 c.c. toxin 1.70 + 1 c.c. serum diluted (1 : 1,000).		+	0
6	1 c.c. toxin 1.70 + 1 c.c. serum diluted (1 : 5,000).		+	±
7	1 c.c. toxin 1.70 + 1 c.c. serum diluted (1 : 10,000)		+	+
8	1 c.c. toxin 1.70 + 1 c.c. sodium chloride		+	+

The tubes were kept for 1 hr. at 37° C. and 24 hrs. on ice.

Antihemolytic Power.—(Table VI.) This and many similar experiments indicate that the serum of an animal immunized by the injection of toxin possesses an increased antihemolytic power. This antihemolytic action is not highly specific, however, as regards the type of organisms; a serum produced by the injection of toxin prepared from pneumococci of type I protects almost as well against a toxin prepared from organisms of type II as against one prepared from pneumococci of homologous type.

In immunization in this manner, the possibility cannot be excluded that in addition to the introduction of the toxin living organisms have also been introduced. It was therefore important to know whether or not an immune serum produced by the injection of living organisms would or would not have an effect on inhibiting the action of the hemolytic toxin. For this purpose the sera of horses immunized by the injection of living pneumococci were studied.

(*c*) *Immune Horse Serum.*—The horses from which these sera were obtained were immunized by injecting intravenously repeated and increasing numbers of living pneumococci previously washed

in salt solution. These bacteria were obtained by centrifugalization from bouillon cultures. Usually one or two doses of pneumococci killed by heat were injected before proceeding to the injection of living bacteria. The sera studied were those that have been used in the treatment of patients and were active in protection only against the organisms of the type used in immunization. The sera were actively and specifically agglutinating.

Serum I had such protective power that when 0.2 of a cubic centimeter of the serum was mixed with 0.1 of a cubic centimeter of a twenty-hour bouillon culture of organisms of type I and the mixture was injected into a mouse, the mouse lived; whereas 0.000001 of a cubic centimeter of the culture injected alone killed within twenty-four hours.

Serum II had a little less protective power, in that 0.2 of a cubic centimeter of serum protected against only 0.01 of a cubic centimeter of culture of which 0.000001 of a cubic centimeter injected alone killed within twenty-four hours.

Tests of the antihemolytic power gave results similar to those obtained in an experiment of which the following is the protocol (table VII).

The study of these sera showed that they possessed high neutralizing power for the hemolytic poison obtained from the bodies of pneumococci, even higher than that present in the serum of rabbits or sheep injected with the toxin. This antihemolytic power, however, is not very specific as regards type of organisms, serum I protecting against the toxin prepared from organisms of type II almost as well as against that prepared from organisms of type I and *vice versa*. That this protective action is not merely a non-specific reaction of all immune sera, however, is shown by the fact that an anti-influenzal serum, kindly supplied by Dr. Wollstein,[11] possessed little or no greater antihemolytic power than did normal serum. In the experiment above described, the anti-influenzal serum had a little greater effect than did normal horse serum, but it was no greater than that of other normal horse sera tested at other times.

(*d*) *Antihemolytic Action of the Serum of Patients Sick of Pneu-*

[11] Wollstein, M., *Jour. Exper. Med.*, 1911, xiv, 73.

TABLE VII.

Toxin 1.70 Prepared from Pneumococci of Type I.

Experiments of October 7, 1913.			Hemolysis			
			Immune horse serum I.	Immune horse serum II.	Normal horse serum.	Anti-influenzal serum.
Toxin (2 hemolytic units) + serum 0.1 c.c.	½ hr. at 37° C.	Sheep corpuscles 0.5 c.c.	0	0	+	0
Toxin (2 hemolytic units) + serum 0.02 c.c.		Sheep corpuscles 0.5 c.c.	0	0	+	±
Toxin (2 hemolytic units) + serum 0.01 c.c.		Sheep corpuscles 0.5 c.c.	0	0	+	+
Toxin (2 hemolytic units) + serum 0.002 c.c.		Sheep corpuscles 0.5 c.c.	0	0	+	+
Toxin (2 hemolytic units) + serum 0.001 c.c.		Sheep corpuscles 0.5 c.c.	0	±	+	+
Toxin (2 hemolytic units) + serum 0.0002 c.c.		Sheep corpuscles 0.5 c.c.	+	+	+	+

Toxin A69, Prepared from Pneumococci of Type II.

Toxin (2 hemolytic units) + serum 0.1 c.c.	½ hr. at 37° C.	Sheep corpuscles 0.5 c.c.	0	0	+	0
Toxin (2 hemolytic units) + serum 0.02 c.c.		Sheep corpuscles 0.5 c.c.	0	0	+	+
Toxin (2 hemolytic units) + serum 0.01 c.c.		Sheep corpuscles 0.5 c.c.	0	0	+	+
Toxin (2 hemolytic units) + serum 0.002 c.c.		Sheep corpuscles 0.5 c.c.	0	0	+	+
Toxin (2 hemolytic units) + serum 0.001 c.c.		Sheep corpuscles 0.5 c.c.	0	±	+	+
Toxin (2 hemolytic units) + serum 0.0002 c.c.		Sheep corpuscles 0.5 c.c.	+	+	+	+

The tubes were kept at 37° C. for 1 hr. and 24 hrs. on ice.

monia and of Those Convalescent from That Disease.—The sera of patients suffering with pneumonia and those of patients during convalescence from this disease have been tested for antihemolytic power against the pneumococcus toxin. It has been impossible, however, to demonstrate that these sera possess an increased antihemolytic action over the controls with normal human serum and the serum of patients suffering from other diseases.

From these studies of the antihemolytic action of immune sera, it is evident that by the injection into rabbits and sheep of a solution containing the bacterial substance of pneumococci, the serum of these animals acquires an increased power of inhibiting the hemolytic action of such a solution. This change in the serum occurs

when the fluid injected consists of solutions of the bacterial bodies in sodium cholate or of solutions prepared by freezing the bacteria and grinding them in salt solution. The antihemolytic power of these sera is not so great, however, as that of sera produced by the injection of living organisms. The latter sera, however, possess marked agglutinating properties, while the former sera have no power of agglutination. While in the production of antitoxic sera the possibility of the injection of a few living organisms cannot be excluded, the lack of agglutinating power renders it extremely probable that the development of antihemolytic properties is due to the injection of the hemolytic substance, and that, therefore, this hemolytic solution possesses antigenic properties and may be considered a true toxin.

SUMMARY.

Solutions of the bodies of pneumococci, obtained by dissolving them in dilute solutions of sodium cholate, by permitting them to undergo autolysis, or by first freezing, drying, and then grinding in salt solution, are actively hemolytic for rabbit, sheep, guinea pig, and human red blood corpuscles. The substance on which this hemolytic property depends is very labile, much of its activity is lost on passing through a filter, and it is destroyed by the action of trypsin. In its properties it corresponds to the substance contained in such solutions which causes the death of guinea pigs on intravenous injection. Its activity is prevented by the presence of minute amounts of cholesterin.

Following the injection of this solution into rabbits and sheep, the sera of these animals acquire increased power of inhibiting its hemolytic action. It therefore possesses antigenic properties.

It may therefore be concluded that the bodies of pneumococci contain a toxin that is hemolytic for red blood corpuscles. This substance is not simply a product of autolysis but undoubtedly exists preformed in the bacterial cell. However, it is not given up to the surrounding fluid as long as the bodies of the bacteria are intact. It may therefore be considered a hemolytic endotoxin.

THE PRODUCTION OF METHEMOGLOBIN BY PNEUMOCOCCI.*

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When pneumococci are grown in media containing blood or hemoglobin, the red color of the latter is changed to a greenish brown, as may be well seen in blood agar plates on which pneumococci are growing. This change is undoubtedly due to the formation of methemoglobin. Butterfield and Peabody[1] have demonstrated that methemoglobin is formed when pneumococci are cultivated in media containing blood.

A similar change in color, though not so pronounced, is seen in the blood of animals dying of acute pneumococcus septicemia, and to a still less extent in the blood of patients severely ill and dying of pneumonia. Peabody[2] has shown that during the terminal stages of fatal cases of pneumonia there occurs a progressive decrease in the oxygen content and the oxygen-combining capacity of the blood. This is evidently due to the transformation of oxyhemoglobin into methemoglobin. A similar change occurs in the blood of rabbits severely infected with pneumococci.[3] While the presence of methemoglobin in artificial culture media may be readily demonstrated by spectroscopic methods, this is more difficult in the blood of patients and infected animals, since a considerable concentration of methemoglobin is necessary for spectroscopic demonstration.

It has seemed of importance to learn more of the nature of the reaction during which methemoglobin is formed by pneumococci, and the present paper gives a report of this study, as far as it has been carried out.

Peabody stated that the transformation of oxyhemoglobin into

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[1] Butterfield, E. E., and Peabody, F. W., *Jour. Exper. Med.*, 1913, xvii, 587.

[2] Peabody, F. W., *Jour. Exper. Med.*, 1913, xviii, 7.

[3] Peabody, F. W., *Jour. Exper. Med.*, 1913, xviii, 1.

methemoglobin is brought about by the filtrates of pneumococcus cultures as well as by the cultures containing bacteria; but our experiments, which have been repeated many times, make it evident that in the experiments on which this statement was based faulty filters were employed. If the bacteria are all removed from a culture fluid, either by centrifugalization or by filtration through a Berkefeld filter, and then blood be added to the filtrate, no formation of methemoglobin occurs. The reaction therefore cannot depend merely upon the production of an acid reaction in the fluid in which the bacteria grow. This is also shown by careful neutralization of the culture before the addition of blood, when the rate of reaction is not changed unless to be increased.

Boiling a broth culture, or even heating it to 56° C. for one half hour, before addition of blood prevents the reaction from occurring. That this inhibition of the reaction by heat depends upon the destruction of the bacteria is rendered probable by the following experiment. A series of tubes containing broth cultures of different races of pneumococci were heated one half hour at 45° C.,—about the thermal death point for most races of pneumococci. Blood corpuscles were then added to each tube and transplantations were made from each tube on fresh media. In the cultures from which growth in the transplantation was obtained the formation of methemoglobin occurred. In the cultures, on the other hand, from which no growth occurred and in which the bacteria were therefore all killed, no formation of methemoglobin took place.

While extracts of pneumococcal bodies cause lysis of red blood corpuscles,[4] they never cause the formation of methemoglobin, unless they contain living pneumococci. The fact that small amounts of sodium cholate added to broth cultures of pneumococci inhibit the formation of methemoglobin might explain the failure of extracts of pneumococci in cholate solution to produce methemoglobin, but a similar failure to form methemoglobin is seen when the extracts are prepared by freezing and grinding the bacteria without the addition of cholate.

These experiments indicate that for the formation of methemoglobin the presence of living bacteria is necessary.

[4] Cole, R., *Jour. Exper. Med.*, 1914, xx, 346.

TABLE I.

Tube No.	Experiments.	Methemoglobin formation. 1 hr. at 37° C.	Methemoglobin formation. 1 hr. at 37° C. and 24 hrs. on ice.
1	1 c.c. broth culture A8/o/6 + 1 c.c. sodium chloride solution + 0.5 c.c. emulsion sheep corpuscles	+ in 20 min.	+
2	1 c.c. broth culture A8/o/6 (1 : 2) + 1 c.c. sodium chloride solution + 0.5 c.c. emulsion sheep corpuscles	+ in 20 min.	+
3	1 c.c. broth culture A8/o/6 (1 : 4) + 1 c.c. sodium chloride solution + 0.5 c.c. emulsion sheep corpuscles	+ in 20 min.	+
4	1 c.c. broth culture A8/o/6 (1 : 8) + 1 c.c. sodium chloride solution + 0.5 c.c. emulsion sheep corpuscles	+ in 20 min.	+
5	1 c.c. broth culture A8/o/6 (1 : 16) + 1 c.c. sodium chloride solution + 0.5 c.c. emulsion sheep corpuscles	0	0
6	1 c.c. emulsion A + 1 c.c. sodium chloride solution + 0.5 c.c. emulsion sheep corpuscles	+ in 20 min.	+
7	1 c.c. emulsion A (1 : 2) + 1 c.c. sodium chloride solution + 0.5 c.c. emulsion sheep corpuscles	+ in 20 min.	+
8	1 c.c. emulsion A (1 : 4) + 1 c.c. sodium chloride solution + 0.5 c.c. emulsion sheep corpuscles	+	+
9	1 c.c. emulsion A (1 : 8) + 1 c.c. sodium chloride solution + 0.5 c.c. emulsion sheep corpuscles	+	+
10	1 c.c. emulsion A (1 : 16) + 1 c.c. sodium chloride solution + 0.5 c.c. emulsion sheep corpuscles	0	0
11	1 c.c. emulsion B + 1 c.c. sodium chloride solution + 0.5 c.c. emulsion sheep corpuscles	0	0
12	1 c.c. emulsion B (1 : 2) + 1 c.c. sodium chloride solution + 0.5 c.c. emulsion sheep corpuscles	0	0
13	1 c.c. emulsion B (1 : 4) + 1 c.c. sodium chloride solution + 0.5 c.c. emulsion sheep corpuscles	0	0
14	1 c.c. emulsion B (1 : 8) + 1 c.c. sodium chloride solution + 0.5 c.c. emulsion sheep corpuscles	0	0
15	1 c.c. emulsion B (1 : 16) + 1 c.c. sodium chloride solution + 0.5 c.c. emulsion sheep corpuscles	0	0

It was now important to learn whether or not washed pneumococci in salt solution could produce the reaction or whether the presence of nutrient medium also is required. That the latter is necessary is shown by the protocol of an experiment which follows. Two portions of a twenty-hour broth culture of pneumococcus A8/o/6 were centrifugalized, the supernatant fluid was removed, and each sediment thoroughly washed in sodium chloride solution and again centrifugalized. One portion of the sediment was now made up to the original volume in broth (emulsion A), and the other portion was made up to the original volume in sodium chloride solution (emulsion B) and tests were made to determine the methemoglobin-producing power (table I) of the two solutions in varying dilutions, compared with the methemoglobin-producing power of an untreated broth culture.

It was suggested that the failure of the reaction to occur in salt solution might be due to the toxicity of sodium chloride in the absence of other inorganic salts. To test this, experiments were carried out with organisms suspended in Ringer solution. No reaction, however, occurred. It is evident, therefore, that some organic constituents of the broth are necessary for the reaction.

Experiments were then made to determine which class of organic substances in broth is essential. The following is a protocol of one such experiment (table II).

While experiments like those in table II showed that the reaction occurs when either sugar, peptone, or protein is present, further studies have shown that while protein substances must be present in quite high concentration in order that the reaction may occur, at least one part of a 10 per cent. solution of crystallized egg albumin to twenty parts of salt solution being required, the reaction occurs with great rapidity when sugar in very great dilution is present, even in dilutions as great as one part of a 5 per cent. dextrose solution to 10,000 parts of salt solution. The presence of traces of sugar in peptone solution and even in solutions of egg albumin can only with great difficulty be excluded, and it is possible that upon the presence of such traces of sugar the availability of these solutions in this reaction depends, since only extremely small amounts of sugar are required.

Various kinds of sugar were next tested to determine whether

TABLE II.

Tube No.	Experiments of December 13, 1913	Methemoglobin formation. 1 hr. at 37° C.	Methemoglobin formation. 24 hrs. on ice.
1	1.5 c.c. sodium chloride solution + 0.5 c.c. hemoglobin solution + 0.5 c.c. emulsion A8	0	0
2	1 c.c. sterile broth + 0.5 c.c. sodium chloride solution + 0.5 c.c. hemoglobin solution + 0.5 c.c. emulsion A8	+ in 10 min.	+
3	1 c.c. sterile broth (1 : 2) + 0.5 c.c. sodium chloride solution + 0.5 c.c. hemoglobin solution + 0.5 c.c. emulsion A8	+ in 10 min.	+
4	1 c.c. sterile broth (1 : 4) + 0.5 c.c. sodium chloride solution + 0.5 c.c. hemoglobin solution + 0.5 c.c. emulsion A8	+ in 10 min.	+
5	1 c.c. sterile broth (1 : 8) + 0.5 c.c. sodium chloride solution + 0.5 c.c. hemoglobin solution + 0.5 c.c. emulsion A8	+ in 10 min.	+
6	1 c.c. sterile broth (1 : 16) + 0.5 c.c. sodium chloride solution + 0.5 c.c. hemoglobin solution + 0.5 c.c. emulsion A8	+ in 10 min.	+
7	1 c.c. 5% dextrose + 0.5 c.c. sodium chloride solution + 0.5 c.c. hemoglobin solution + 0.5 c.c. emulsion A8	+ in 10 min.	+
8	1 c.c. 5% dextrose (1 : 2) + 0.5 c.c. sodium chloride solution + 0.5 c.c. hemoglobin solution + 0.5 c.c. emulsion A8	+ in 10 min.	+
9	1 c.c. 5% dextrose (1 : 4) + 0.5 c.c. sodium chloride solution + 0.5 c.c. hemoglobin solution + 0.5 c.c. emulsion A8	+ in 10 min.	+
10	1 c.c. 5% dextrose (1 : 8) + 0.5 c.c. sodium chloride solution + 0.5 c.c. hemoglobin solution + 0.5 c.c. emulsion A8	+ in 10 min.	+
11	1 c.c. 5% dextrose (1 : 16) + 0.5 c.c. sodium chloride solution + 0.5 c.c. hemoglobin solution + 0.5 c.c. emulsion A8	+ in 10 min.	+
12	1 c.c. 5% peptone + 0.5 c.c. sodium chloride solution + 0.5 c.c. hemoglobin solution + 0.5 c.c. emulsion A8	+ in 10 min.	+
13	1 c.c. 5% peptone (1 : 2) + 0.5 c.c. sodium chloride solution + 0.5 c.c. hemoglobin solution + 0.5 c.c. emulsion A8	+ in 10 min.	+
14	1 c.c. 5% peptone (1 : 4) + 0.5 c.c. sodium chloride solution + 0.5 c.c. hemoglobin solution + 0.5 c.c. emulsion A8	+ in 10 min.	+
15	1 c.c. 5% peptone (1 : 8) + 0.5 c.c. sodium chloride solution + 0.5 c.c. hemoglobin solution + 0.5 c.c. emulsion A8	+ in 10 min.	+
16	1 c.c. 5% peptone (1 : 16) + 0.5 c.c. sodium chloride solution + 0.5 c.c. hemoglobin solution + 0.5 c.c. emulsion A8	+ in 10 min.	+
17	1 c.c. 4% egg albumin + 0.5 c.c. sodium chloride solution + 0.5 c.c. hemoglobin solution + 0.5 c.c. emulsion A8	+ in 10 min.	+
18	1 c.c. 4% egg albumin (1 : 2) + 0.5 c.c. sodium chloride solution + 0.5 c.c. hemoglobin solution + 0.5 c.c. emulsion A8	+ in 10 min.	+
19	1 c.c. 4% egg albumin (1 : 4) + 0.5 c.c. sodium chloride solution + 0.5 c.c. hemoglobin solution + 0.5 c.c. emulsion A8	+ in 10 min.	+
20	1 c.c. 4% egg albumin (1 : 8) + 0.5 c.c. sodium chloride solution + 0.5 c.c. hemoglobin solution + 0.5 c.c. emulsion A8	+ in 10 min.	+
21	1 c.c. 4% egg albumin (1 : 16) + 0.5 c.c. sodium chloride solution + 0.5 c.c. hemoglobin solution + 0.5 c.c. emulsion A8	+ in 10 min.	+
22	1 c.c. sterile broth + 1 c.c. sodium chloride solution + 0.5 c.c. hemoglobin solution	0	0
23	1 c.c. 5% dextrose + 1 c.c. sodium chloride solution + 0.5 c.c. hemoglobin solution	0	0
24	1 c.c. 5% peptone + 1 c.c. sodium chloride solution + 0.5 c.c. hemoglobin solution	0	0
25	1 c.c. 4% egg albumin + 1 c.c. sodium chloride solution + 0.5 c.c. hemoglobin solution	0	0

The hemoglobin solution = 2 c.c. washed sheep corpuscles plus 8 c.c. distilled water plus 30 c.c. 0.85 per cent. sodium chloride solution.

Dextrose = 5 per cent. solution of chemically pure dextrose in distilled water, autoclaved for 20 minutes.

Peptone = 5 per cent. solution of Witte's peptone in distilled water, autoclaved for 20 minutes.

Egg albumin = 4 per cent. solution of crystallized egg albumin in distilled water.

Bacterial emulsion = emulsion in sodium chloride solution of washed sediment of 20-hour broth culture A8. The bacteria were in twice as great concentration as in the culture.

pneumococci cause the formation of methemoglobin only in the presence of dextrose or whether this sugar may be replaced by others in which the molecular configuration is different. Several races of pneumococci as well as streptococci were employed and the results are given in table III.

In another experiment *d*-xylose and *d*-arabinose were also tested. The results are given in table IV.

While in the experiments given no reaction occurred in the presence of inulin, in several other experiments a slight reaction occurred in solutions containing 2 per cent. of this sugar. In these and many other experiments as well, however, no reaction ever occurred in the solutions containing ribose. It is therefore apparent that while the reaction occurs in the presence of most sugars, the configuration of the molecule makes some difference. This is also seen in the effect of different sugars on the rate of reaction. The reaction always occurred more slowly with saccharose and arabinose than with the other sugars. Since the reaction occurs when pneumococci are placed in solutions containing traces of sugar, and it is known that pneumococci cause breaking down of many sugars, the possibility suggested itself that the reaction is due to some substance formed during the decomposition of the sugar molecule. The reaction cannot, however, be due simply to the formation of carbon dioxide. If carbon dioxide be passed through a solution of oxyhemoglobin the solution becomes cherry red, and if oxygen now be passed through it again becomes bright red. Such changes never occur in solutions of hemoglobin acted upon by pneumococci. A similar change is well seen when yeast is added to solutions containing blood corpuscles. In this case the blood takes on a magenta color which on shaking becomes bright red. It would appear possible, however, that the change might be due to some intermediate product of sugar metabolism. The exact transformation which the sugar molecule undergoes during the process of changing into carbon dioxide and water is still obscure, but there are several substances which are thought to represent intermediate stages. A series of such substances which are thought to be intermediate products in the metabolism of sugar and also certain ones that are known to be end products under certain conditions were tested. These substances

TABLE III.

Experiments.	Methemoglobin formation. A18	Methemoglobin formation. P1.77	Methemoglobin formation. M1.77	Methemoglobin formation. Streptococcus.
1 c.c. 2% ribose solution + 1 c.c. bacterial emulsion + 1 c.c. hemoglobin solution......	0	0	0	0
1 c.c. 2% arabinose solution + 1 c.c. bacterial emulsion + 1 c.c. hemoglobin solution....	+ in 1 hr.	+ in 10 min.	+ in 1 hr.	0
1 c.c. 2% xylose solution + 1 c.c. bacterial emulsion + 1 c.c. hemoglobin solution......	+ in 10 min.	+ in 10 min.	+ in 10 min.	0
1 c.c. 2% maltose solution + 1 c.c. bacterial emulsion + 1 c.c. hemoglobin solution.....	+ in 1 hr.	+ in 10 min.	+ in 10 min.	0
1 c.c. 2% lactose solution + 1 c.c. bacterial emulsion + 1 c.c. hemoglobin solution.....	+ in 1 hr.	+ in 10 min.	+ in 10 min.	0
1 c.c. 2% saccharose solution + 1 c.c. bacterial emulsion + 1 c.c. hemoglobin solution ...	+ in 1 hr.	+ in 1 hr.	+ in 1 hr.	0
1 c.c. 2% dextrose solution + 1 c.c. bacterial emulsion + 1 c.c. hemoglobin solution....	+ in 10 min.	+ in 10 min	+ in 10 min.	0
1 c.c. 2% levulose solution + 1 c.c. bacterial emulsion + 1 c.c. hemoglobin solution....	+ in 10 min.	+ in 10 min.	+ in 10 min.	0
1 c.c. 2% mannose solution + 1 c.c. bacterial emulsion + 1 c.c. hemoglobin solution....	+ in 10 min.	+ in 10 min.	+ in 10 min.	0
1 c.c. 2% galactose solution + 1 c.c. bacterial emulsion + 1 c.c. hemoglobin solution....	+ in 10 min.	+ in 10 min.	+ in 10 min.	0
1 c.c. 2% inulin solution + 1 c.c. bacterial emulsion + 1 c.c. hemoglobin solution......	0	0	0	0
1 c.c. 2% raffinose solution + 1 c.c. bacterial emulsion + 1 c.c. hemoglobin solution	+ in 10 min.	+ in 10 min.	+ in 10 min.	0

The tubes were all kept at 37° C.

A18, P1.77, and M1.77 are pneumococci of type I. The streptococcus is a virulent hemolytic streptococcus.

TABLE IV.

Experiments.	Methemoglobin formation.		
	A19/5/7	A26/0/3	I/73
1 c.c. ribose...........2 % solution + 1 c.c. bacterial emulsion + 0.5 c.c. hemoglobin solution	0	0	0
1 c.c. *l*-arabinose.......2 % solution + 1 c.c. bacterial emulsion + 0.5 c.c. hemoglobin solution	+ in 15 min.	+ in 15 min.	+ in 15 min.
1 c.c. *d*-arabinose......2 % solution + 1 c.c. bacterial emulsion + 0.5 c.c. hemoglobin solution	0	0	0
1 c.c. *l*-xylose..........2 % solution + 1 c.c. bacterial emulsion + 0.5 c.c. hemoglobin solution	+ in 15 min.	+ in 15 min.	+ in 15 min.
1 c.c. *d*-xylose.........2 % solution + 1 c.c. bacterial emulsion + 0.5 c.c. hemoglobin solution	0	0	0
1 c.c. dextrose.........2 % solution + 1 c.c. bacterial emulsion + 0.5 c.c. hemoglobin solution	+ in 15 min.	+ in 15 min.	+ in 15 min
1 c.c. mannose.........2 % solution + 1 c.c. bacterial emulsion + 0.5 c.c. hemoglobin solution	+ in 15 min.	+ in 15 min.	+ in 15 min.
1 c.c. inulin...........2 % solution + 1 c.c. bacterial emulsion + 0.5 c.c. hemoglobin solution	0	0	0

The tubes were kept for 2 hours at 37° C.

were kindly supplied by Dr. G. M. Meyer. They were methylglyoxal, acetaldehyde, pyruvic acid, formaldehyde, formic acid, and acetic acid. Methylglyoxal, acetaldehyde, formaldehyde, and pyruvic acid produced methemoglobin only when very concentrated solutions were employed. The presence of bacteria did not increase their effect. Since such extremely small amounts of sugar are required in the formation of methemoglobin by bacteria, it is not possible that under these conditions any of the substances are present in a concentration sufficient to bring about the reaction, unless such substances are much more active in a nascent state than they are in solution.

The tests with formic acid showed that this substance caused the formation of methemoglobin only when it was used undiluted or but slightly diluted. Even under these conditions the reaction is due to the acidity of the solution, since, after neutralization and the addition of a mixture of primary and secondary potassium phosphates, in order to keep the mixture neutral, no reaction occurred, even with the undiluted acid. When a solution of formic acid, not in itself able to produce methemoglobin, is added to the bacterial emulsion plus hemoglobin solution, no formation of methemoglobin occurs, while the addition of a very dilute solution of dextrose under similar conditions causes the reaction to occur within a few minutes.

Similar results were obtained when acetic acid was used.

These studies, therefore, did not lead to the detection of any substance formed during sugar metabolism or fermentation to which the change of oxyhemoglobin into methemoglobin could be ascribed.

Other substances, such as alcohols, were also tested as to their effect in the formation of methemoglobin. The following results were obtained (table V).

None of these substances, except glycerin, when employed in low dilutions are able to replace dextrose in the mixtures in which methemoglobin is formed. When, however, glycerin, even in a dilution of 1 to 10,000, is added, the formation of methemoglobin occurs rapidly.

A large number of quantitative studies with these and similar substances were made, in the hope of finding some relationship in chemical structure between those substances which by their presence per-

TABLE V.

Experiments.	Results.
2 c.c. glycerin ....(10 % solution) + 0.5 c.c. hemoglobin solution	No change in 24 hrs.
2 c.c. mannite ....(10 % solution) + 0.5 c.c. hemoglobin solution	No change in 24 hrs.
2 c.c. methyl alcohol ....(10 % solution) + 0.5 c.c. hemoglobin solution	No change in 24 hrs.
2 c.c. ethyl alcohol ....(10 % solution) + 0.5 c.c. hemoglobin solution	No change in 24 hrs.
1 c.c. bacterial emulsion + 1 c.c. glycerin ....(1 : 10,000) + 0.5 c.c. hemoglobin solution	+ in 10 min.
1 c.c. bacterial emulsion + 1 c.c. mannite ....(10 % solution) + 0.5 c.c. hemoglobin solution	+ in 3 hrs.
1 c.c. bacterial emulsion + 1 c.c. methyl alcohol ....(10 % solution) + 0.5 c.c. hemoglobin solution	+ in 3 hrs.
1 c.c. bacterial emulsion + 1 c.c. ethyl alcohol ....(10 % solution) + 0.5 c.c. hemoglobin solution	+ in 2 hrs.
1 c.c. bacterial emulsion + 1 c.c. dextrose ....(5 % solution) + 0.5 c.c. hemoglobin solution	+ in 10 min.
1 c.c. bacterial emulsion + 1 c.c. starch ....(1 % solution) + 0.5 c.c. hemoglobin solution	+ in 1 hr.
1 c.c. bacterial emulsion + 1 c.c. urea ....(2 % solution) + 0.5 c.c. hemoglobin solution	No change in 24 hrs.
1 c.c. bacterial emulsion + 1 c.c. ethyl acetate ....(0.2 % solution) + 0.5 c.c. hemoglobin solution	+ in 1 hr.
1 c.c. bacterial emulsion + 1 c.c. leucin ....(2 % solution) + 0.5 c.c. hemoglobin solution	No change in 24 hrs.
1 c.c. bacterial emulsion + 1 c.c. cholesterin ....(1 % solution) + 0.5 c.c. hemoglobin solution	No change in 24 hrs.

mit pneumococci to form methemoglobin. So large a number of substances are suitable for the purpose that it has not been possible to show that any form of molecular configuration or grouping is essential.

It seems probable, judging from these studies, that the formation of methemoglobin occurs whenever pneumococci in contact with hemoglobin are able to carry on metabolic and functional activities, probably including multiplication, and that this happens when traces of sugar and also when large amounts of other organic substances are present. While in the absence of those substances death of the bacteria does not necessarily occur, it is probable that their metabolic activities are reduced to a minimum.

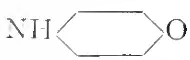
Cellular metabolic processes occur through the agency of ferments contained within the cells, and it would therefore seem probable, if the reaction under consideration were due to the active processes of oxidation occurring in the medium immediately surrounding the bacterial cells, that similar reactions would appear when bacterial extracts are used instead of the living bacteria. As Warburg[5] has pointed out, however, the reactions which occur in the living cell are not identical, at least in intensity, with those induced by the ferments when removed from the cell by extraction or other means. The activity of zymase, for instance, is markedly less than that of a corresponding number of living yeast cells. The importance of structure in cellular activity cannot be neglected, and from the fact that the reaction under consideration is not induced by the presence of bacterial extracts, it does not necessarily follow that the reaction is not due to the functional activities of the bacterial cells.

Studies concerning the formation of methemoglobin from oxyhemoglobin by means of chemicals indicate that the reaction is of the nature of an oxidation. The exact chemical nature of oxyhemoglobin as well as that of methemoglobin has not been absolutely determined. It is not even definitely known whether the methemoglobin molecule contains an amount of oxygen equal to or less than that of the oxyhemoglobin molecule. There is considerable evidence,[6] however, for the view that while in oxyhemoglobin the

[5] Warburg, O., Ueber die Wirkung der Struktur auf chemische Vorgänge in Zellen, Jena, 1913.

[6] von Reinhold, B., *Ztschr. f. physiol. Chem.*, 1913, lxxxv, 250.

oxygen is loosely combined as in an oxide, the formula being usually written $\mathrm{Hb}{<}^{O}_{O}$, in methemoglobin the oxygen is as in an hydroxide. Whether the molecule contains one hydroxyl group as in Hb–OH, or two hydroxyl groups as in $\mathrm{Hb}{<}^{OH}_{OH}$, is not determined, though there is experimental evidence for both points of view. In either case, however, methemoglobin would represent a lower stage of oxidation than oxyhemoglobin, and the transformation of oxyhemoglobin into methemoglobin would therefore be of the nature of a reduction.

On the other hand evidence has been presented by Heubner[7] to show that the formation of methemoglobin is always an oxidation process. As is well known, substances which are known to be oxidizing agents and also those considered to be reducing agents may bring about the transformation of oxyhemoglobin into methemoglobin. An explanation of this fact, which Heubner presents, is that the reducing agents are first oxidized, this oxidation occurring better in the presence of oxyhemoglobin, and then reduced, giving up their oxygen to form methemoglobin. It is of great interest that certain substances, such as aminophenol, , are able to convert much more hemoglobin into methemoglobin than could occur if the reaction were a simple molecular one. One molecule of aminophenol may apparently transform at least fifty molecules of hemoglobin into methemoglobin. The aminophenol must therefore repeatedly react with the hemoglobin. Heubner's view is that the aminophenol is first oxidized to a quinone, (quinonimine, ) and then again reduced to aminophenol, in this reaction the hemoglobin being changed to methemoglobin. The reaction may be repeated many times, the aminophenol therefore having a catalytic-like action.

If this be the true explanation of the kind of reaction occurring in the transformation of oxyhemoglobin into methemoglobin by organic chemical substances, it is possible that the mode of action of pneumococci in causing the formation of methemoglobin may fol-

[7] Heubner, B., *Arch. f. exper. Path. u. Pharmakol.*, 1913, lxxii, 239.

low similar lines. The metabolic activities of cells consist largely of oxidative and reduction processes. When oxyhemoglobin is brought into contact with pneumococci it may be changed into methemoglobin, because it is then exposed to active oxidative and reduction processes occurring in the neighborhood of the bacterial cells. These processes must be of a special type in the case of pneumococci, however, since otherwise all living cells would cause the transformation.

That this change goes on in the neighborhood of the cells and not necessarily in the cells themselves is shown by the fact that while the reaction occurs more readily when the hemoglobin is in solution in the medium surrounding the bacteria, it may also occur when the hemoglobin is contained within red blood corpuscles, the blood cells and bacteria being merely in intimate contact. If, however, the bacteria and blood cells are separated by a membrane of any kind, even a very thin layer of oil, the reaction does not occur.

If the change of hemoglobin into methemoglobin taking place in the neighborhood of pneumococcal cells is due to oxidative processes occurring there, it was thought that evidence for this fact would be obtained by studying the effect which the presence or absence of free oxygen would have on the reaction. This was done by testing the rate of reaction under the following conditions: To remove oxygen from a solution, a stream of hydrogen was passed through it for ten minutes, the entrance tube passing completely to the bottom of the tube containing the solution. The solution was then covered by a layer of paraffin oil. Passing hydrogen in this way through a solution of hemoglobin causes the solution to take on a dark color due to the formation of reduced hemoglobin. In mixing two solutions so treated, a pipette was passed through the paraffin layer of one to the bottom of the tube and the fluid was drawn into the pipette and added to the second solution, by plunging the tip of the pipette through the oil on the surface. Care was taken not to empty the pipette completely, as in this way air would be admitted. While complete absence of oxygen could not be obtained by this method, it was sufficiently excluded for the purpose intended.

It was found that if an emulsion of red blood cells, so treated with hydrogen, was added to a broth culture of pneumococci, also treated with hydrogen, no formation of methemoglobin occurred for some hours. When the tubes stood over night there usually

occurred some change into methemoglobin, usually only at the surface under the layer of oil. If, moreover, such a mixture has stood for some time without the formation of methemoglobin, and oxygen now be bubbled through, the change into methemoglobin occurs with great readiness. It is evident, therefore, that the presence of oxygen is necessary for the reaction.

On the other hand, an excess of free oxygen somewhat delays the reaction. If two tubes are prepared, each containing a mixture of an emulsion of red blood cells and an emulsion of pneumococci, and if oxygen be bubbled through one tube, while the other is simply allowed to stand exposed to the air, the two tubes being kept at the same temperature, the change into methemoglobin occurs more slowly in the tube through which oxygen is passing. To control the possibility that the mechanical disturbance due to the bubbling gas may account for the difference, air was passed through the second tube. The reaction was again delayed in the tube through which oxygen was passing.

Two tubes were now prepared, one tube containing a mixture of an emulsion of red blood cells and an emulsion of bacteria, each of which had been previously saturated with hydrogen, and the other containing an identical mixture except that no hydrogen had been passed through. Oxygen was now bubbled through both tubes, which were kept at the same temperature and under identical conditions. In the tube containing the mixture previously saturated with hydrogen the reaction occurred more rapidly than it did in the other tube.

The above results are briefly shown in the following protocol of one experiment (table VI).

It is now easy to interpret these experiments in the light of

TABLE VI.

Tube No.	Experiments.		Methemoglobin formation.
1	4 c.c. broth culture A82/1/12 + 2 c.c. hemoglobin solution.......		+ in 7 min.
2	4 c.c. broth culture A82/1/12 saturated with hydrogen + 2 c.c. hemoglobin solution saturated with hydrogen		0
3	4 c.c. broth culture A82/1/12 + 2 c.c. hemoglobin solution	Oxygen bubbled through	+ in 28 min.
4	4 c.c. broth culture A82/1/12 saturated with hydrogen + 2 c.c. hemoglobin solution saturated with hydrogen	Oxygen bubbled through	+ in 17 min.

The tubes were all kept at 37° C.

what is known concerning the mode of production of methemoglobin by substances like aminophenol. According to this interpretation the formation of methemoglobin by pneumococci occurs as the result of reduction and oxidative processes occurring in the neighborhood of the bacteria. The oxyhemoglobin is first reduced and if this is inhibited by an excess of free oxygen the reaction is delayed. On the other hand, after reduction has occurred a free supply of oxygen accelerates the reaction. If oxygen be excluded no reaction whatever can occur.

The writer realizes that with the present knowledge it is impossible to conclude that precisely this mode of reaction occurs; but it seems to be the best explanation at present available of the observed experimental facts. It may be objected that the effect of the presence of hydrogen and oxygen is to inhibit or accelerate the metabolic activities of the bacteria rather than to cut off or to increase the supply of oxygen required for the chemical changes. The fact that an excess of oxygen delays the reaction is against this interpretation, though it is known that an excess of oxygen may inhibit cellular action or even be directly toxic to cells.

Experiments were also undertaken to determine whether, in the absence of free oxygen, the oxygen required for the reaction could be obtained from methylene blue, if this be added to the mixture. It was found that the reaction proceeds under these circumstances, but more slowly and less completely than in the presence of free oxygen.

It is believed that the experiments concerning the production of methemoglobin by pneumococci are important not only because they may possibly explain a reaction which probably occurs in every animal severely infected with pneumococci, but they are also important because they suggest a possible explanation for the pathological action of those bacteria which apparently do not produce an active toxin. Since bacteria may injure red blood corpuscles by merely changing oxidative processes in their vicinity, and without producing substances capable of isolation, it is possible that bacteria may injure other tissue cells in a similar manner. Therefore, the pathological effects of bacteria are not necessarily due to the action of a definite poison, but may be due to disturbances in oxidation in the immediate neighborhood of the bacteria.

CONCLUSIONS.

1. Pneumococci in contact with hemoglobin transform this into methemoglobin. This reaction occurs only when the pneumococci are living; it is not induced by the culture fluid or by extracts of the bacteria.

2. The reaction does not occur when hemoglobin is added to an emulsion of washed pneumococci in salt solution. However, if minute traces of dextrose be added to such a mixture, the reaction quickly occurs. The dextrose may be replaced by any one of a number of other sugars, and also by certain other organic substances, if the latter are added in large amounts. Certain other organic substances are not able to replace dextrose, but it has been impossible to determine any special molecular configuration on which this property depends.

3. The formation of methemoglobin by pneumococci probably resembles the formation of methemoglobin by certain chemical substances, such as aminophenol.

4. From the work of others it is probable that the formation of methemoglobin is always a reaction of oxidation. In the formation of methemoglobin by reducing agents, the latter are first oxidized, this occurring better in the presence of oxyhemoglobin. In certain instances an alternate oxidative and reduction of the transforming agent occurs, so that the reaction is continuous.

The effect which the presence or absence of free oxygen has on the reaction with pneumococci suggests that this follows similar lines.

5. The reaction does not occur in the absence of oxygen. If the free oxygen be first removed, and then replaced, the reaction occurs more rapidly than if the oxygen had not been removed. The presence of free oxygen in excess slightly delays the reaction, possibly because of the inhibition of the reduction process which forms the first part of the reaction.

6. The explanation of this phenomenon of methemoglobin production is not only of importance so far as this special reaction is concerned, but also because it suggests an explanation for the manner in which pathological effects are produced by those bacteria which apparently produce no soluble toxin.

THE RÔLE OF THE SPLEEN IN BLOOD FORMATION.*

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Various authors[1] have described the appearance in the spleen in the course of chronic anemias of abundant cells which give evidence of the new formation of blood in that organ when blood formation is greatly needed by the body. These cells are erythroblasts and mononuclear cells of various sizes which become very abundant in the venous sinuses. The same cells are found in the capillaries of the liver and occasionally outside them in the periportal tissues, but not in great numbers in the circulating blood.

The information derived from attempting to estimate the change in the numbers of cells in such an organ as the spleen was felt to be unsatisfactory and a method of study other than the staining of sections of the fixed spleen was sought. Without any knowledge of previous efforts in this direction it was thought that accurate counts of the blood leaving the spleen by the splenic vein when compared with similar counts of that entering by the splenic artery should give a clear idea of the activity of the spleen with respect to its contribution of cells to the blood. Since the completion of the work a few references to similar studies have been found, but it nevertheless seems that a detailed statement of the result of these counts would be of value, especially since the method which is so useful has been employed by hardly any modern workers.

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[1] Bizzozero, G., and Salvioli, G., *Arch. p. le sc. med.*, 1880, iv, 49. Meyer, E., and Heineke, A., *Verhandl. d. deutsch. path. Gesellsch.*, 1906, ix, 224; *Deutsch. Arch. f. klin. Med.*, 1907, lxxxviii, 435. Morris, R. S., *Bull. Johns Hopkins Hosp.*, 1907, xviii, 200. von Domarus, A., *Arch. f. exper. Path. u. Pharmakol.*, 1908, lviii, 319. Heinz, R., *Virchows Arch. f. path. Anat.*, 1902, clxviii, 501. Jolly, J., and Rossello, H., *Compt. rend. Soc. de biol.*, 1909, lxvi, 40. Schridde, H., *Centralbl. f. allg. Path. u. path. Anat.*, 1908, xix, 865. Naegeli, O., Ehrlich, P., and Lazarus, A., Die Anaemie, Vienna and Leipzig, 1909.

It has been known for a long time that the leucocytic count of the blood from the splenic vein exceeds that of the artery.

Vierordt[2] in four counts made on an executed man found the relation of leucocytes to red corpuscles in the splenic vein to be 1 : 49. Hirt[3] found that in the calf the relation was 1 : 60, while in the arterial blood it is 1 : 2,200. Funke[4] gives this relation in the splenic vein as 1 : 4, while Frey[5] calculated the proportion in an old man dead of pneumonia as 1 : 102. Rindfleisch[6] called attention to the richness of the splenic blood in leucocytes especially after a meal. Weidenreich[7] undertook counts from the sinuses of the spleen and obtained variable results inasmuch as some of them contained few or no leucocytes, while others were filled with them doubtless because those sinuses into which the arterial stream enters directly will contain predominantly red corpuscles while the reverse condition will obtain where the lymph channels enter. As a mean I found a proportion of 1 : 15. If we take the relation in the artery (following Hirt) as 1 : 2,200, and the average of the figures from the literature as 1 : 30 it is seen that the splenic vein contains about seventy times as many leucocytes as the efferent artery. Since special efferent lymphatic channels for the drainage of the lymphocytes of the spleen have been shown not to exist, the cells must be discharged into the vena lienalis which thus contains both blood and lymph.

Von Ebner[8] states that it has been shown that the blood of the splenic vein contains an uncommonly large number of white corpuscles and that the site of their formation is the splenic tissue itself from which they enter through the permeable walls of the venules. Loewit[9] studied the relation of leucocytes of arterial blood and splenic venous blood in the guinea pig and found 30 to 80 times as many leucocytes in the venous blood, the increased proportion being due to small and large mononuclear elements. This finding is not obtained in all animals probably because of the irregular or intermittent discharge of the cells. Certain negative results of Tarchanoff and Swaen[10] are probably due to this cause.

The cardinal idea, therefore, in the present study was to examine carefully the blood entering the spleen and to compare it with that emerging by the splenic vein. Any differences should

[2] Vierordt, *Arch. f. physiol. Heilk.*, 1854, xiii, 259, 408.

[3] Hirt, E., *Arch. f. Anat., Physiol., u. wissensch. Med.*, 1856, 174.

[4] Funke, O., Lehrbuch der Physiologie, Leipzig, 1863, i.

[5] Frey, H., Handbuch der Histologie und Histochemie des Menschen, 4th edition, Leipzig, 1874.

[6] Rindfleisch, G. E., Experimentelle Studien ueber die Histologie des Blutes, Leipzig, 1863.

[7] Weidenreich, F., *Arch. f. mikr. Anat.*, 1901, lviii, 247.

[8] von Ebner, V., *Köllikers Handbuch der Gewebelehre*, 1902, iii, 276.

[9] Loewit, M., *Folia Haematol.*, 1907, iv, 473.

[10] Tarchanoff, J., and Swaen, A., *Arch. de physiol. norm. et path.*, 1875, ii, series 2, 324.

represent the changes produced by the spleen. Counts were made with the Thoma-Zeiss apparatus with blood from the splenic artery, splenic vein, mesenteric vein, and, for purposes of control, from a peripheral systemic vein. Smears of blood were also made from each situation. Rabbits, cats, and dogs were studied, some in perfect health, others suffering from spontaneous or artificially induced disease. Thorough autopsies were made in each case and the organs studied microscopically. The counts were made in the usual way but with the most scrupulous attention to technical accuracy in every detail. For the white corpuscles 0.2 per cent. acetic acid was used and for the red cells fresh Toison's solution. Differential counts of the white cells were made in all cases for the blood from each situation.

In securing blood from the splenic artery and vein considerable care must be taken to have a dry field without great loss of blood in the earlier steps of the operation. An incision from the midline along the left costal margin to the axillary line was found to give the best exposure, after which the assistant could elevate the spleen without making traction upon the vessels, by lifting up the stomach and gently drawing the spleen forward so as to expose the hilum. Care was taken not to handle or squeeze the spleen so as to force out its contents artificially. The remaining viscera were protected and the spleen supported by warm cloths so that the circulation proceeded normally. The blood must be obtained from the splenic vein first, as interference with the arterial current will produce changes in that of the veins, while the venous anastomosis and collateral trunks are so numerous that occlusion of one produces no effect.

Some counts were made from blood allowed to spurt from a puncture in the vein, but since this hemorrhage makes it difficult to work later with the artery it was found advisable to put on two Carrel clamps and make the puncture between them. The latter is the more satisfactory procedure since a few drops only may be allowed to flow, and the counts do not differ appreciably from those made from the freely flowing blood.

Ehrlich's triacid stain after fixation by heat and Jenner's, Wright's, and Giemsa's stains after methyl alcohol fixation were

used for the differential counts. Prolonged staining with diluted Giemsa's stain gave good results.

The experiments showed that the blood of the splenic vein differs remarkably from that of the artery not only in the character of its cells but in their number, and this difference was found to exist also between the blood of the splenic vein and that of a peripheral systemic vein.

These differences may be summed up as follows:

(1) The number of red corpuscles per cubic millimeter in the blood of the splenic vein is greater than in that of the artery.

(2) Similarly the number of white corpuscles in the venous blood is greater than in the arterial blood.

(3) The character and proportion of the several varieties of white corpuscles is different in the splenic vein from what is found in the artery. Large mononuclear leucocytes appear in great excess there.

(4) The blood of the splenic vein contains more perfect and larger red corpuscles than that of the artery and they seem richer in hemoglobin.

(5) The blood of the inferior mesenteric veins differs from that of the splenic vein in being relatively richer in small mononuclear cells and poorer in the large mononuclears.

In the tabulated and detailed results given below (table I) these differences are more definitely brought out. It will be noted that the counts made from the ear vein and splenic artery of the same animal correspond very closely, as would be expected, while a marked difference was always found when blood was taken from the splenic veins.

It will be seen from table I that when the autopsy showed the animals operated on to be normal the results were invariably the same. There were always more red and white cells in the splenic veins than could be found in any of the other vessels. Frequently this excess of corpuscles rose to such a degree that there were twice as many red or white cells coming out as going into the spleen. That the excess of white cells should be mainly of the mononuclear variety was to be expected. But there occurred a further noteworthy fact, namely, that these mononuclears were mainly of the

TABLE I.

Animal.	Blood count.	Ear vein.	Splenic artery.	Splenic vein.	Inferior mesenteric vein.	Autopsy.
Rabbit 1	Red blood count	5,400,000	5,200,000	6,700,000		Small nodule of coccidiosis in liver, otherwise normal.
	White blood count	4,000	4,000	9,400		
	Polynuclears	60%	61%	32%		
	Mononuclears	40%	39%	68%		
Rabbit 2	Red blood count	5,900,000	5,700,000	8,600,000		All organs appear normal.
	White blood count	10,600	10,000	25,600		
	Polynuclears	47%	49%	20%		
	Mononuclears	53%	51%	80%		
Rabbit 3	Red blood count	4,600,000	4,560,000	5,360,000		Young rabbit, normal.
	White blood count	5,200	5,000	13,600		
	Polynuclears	30 to 32%	30 to 32%	18 to 20%		
	Mononuclears	68 to 70%	68 to 70%	80 to 82%		
Rabbit 4	Red blood count	Not done	5,280,000	6,800,000		Lactating rabbit. Marked coccidiosis in liver. Spleen small, shrunken, and dark red.
	White blood count	9,800	9,400	4,600	Portal vein	
	Polynuclears	38%	36%	10%	32%	
	Mononuclears	62%	64%	90%	68%	
Dog 1	Red blood count	5,280,000	5,280,000	6,640,000		Normal.
	White blood count	17,000	16,400	34,400	16,400	
	Polynuclears	90%	85%	70%		
	Mononuclears	10%	15%	30%		
Dog 2	Red blood count	4,500,000	4,320,000	6,600,000		Normal.
	White blood count	6,900	7,000	14,800	10,600	
	Polynuclears	61%	60%	44%	45%	
	Mononuclears	39%	40%	56%	55%	
	Large mononuclears			47%	10%	
	Small mononuclears			9%	45%	
Dog 3	Red blood count	5,900,000	5,800,000	7,240,000		All organs normal.
	White blood count	19,700	19,700	19,900		
Dog 4	Red blood count	4,200,000	4,100,000	6,500,000		All organs normal.
	White blood count	20,000	20,000	25,000		
	Polynuclears	80%	81%	70%		
	Mononuclears	20%	19%	30%		
	Large mononuclears	12%	12%	24%		
Cat 1	Red blood count		4,400,000	9,120,000		Apparently normal.
	White blood count		18,000	44,000		
	Polynuclears		75%	30%		
	Mononuclears		25%	70%		

TABLE I.—*Continued.*

Animal	Blood count.	Ear vein.	Splenic artery.	Splenic vein.	Inferior mesenteric vein.	Autopsy.
Cat 2	Red blood count		9,600,000	13,104,000	9,410,000	All organs apparently normal except spleen which is enlarged to about 4 times its normal size and is very mottled on cross-section, due to tremendously enlarged Malpighian bodies. Two accessory spleens presenting same structure. *Microscopical Examination.*—Enormously enlarged follicles. Active mitoses. Huge mononuclear cells.
	White blood count		89,400	137,200	89,400	
	Polynuclears		75 %	74 %	75 %	
	Mononuclears		25 %	26 %	25 %	
	Large mononuclears		25 %	25 %	20 %	
	Small mononuclears		0	1 %	5 %	
Cat 3	Red blood count		7,280,000	11,200,000		Spleen appears normal in size, color, shape, and cross-section. Liver normal. All other organs except mesenteric lymph nodes are normal. Mesenteric lymph nodes especially in duodenal region are enormously enlarged, firm, and matted together in places. Dark red in color. *Microscopical Examination.*—Sections of lymph nodes show tremendous enlargement of germinal centers. Long rod-like bacilli in marginal sinuses. Sections of spleen and liver normal.
	White blood count		26,000	13,500	26,000	
	Polynuclears		18%	40%	19%	
	Mononuclears		82%	60%	81%	
	Large mononuclears		11%	50%	9%	
	Small mononuclears		71%	10%	72%	

TABLE I.—*Concluded.*

Animal.	Blood count.	Left ventricle.	Right auricle.	Splenic vein.	Portal vein.	Autopsy.
Received intraperitoneal inoculation of virulent paratyphoid culture 21 hrs. previously.						
Rabbit 5	Red blood count	4,300,000		6,240,000		Peritonitis. Focal necroses in liver. Large mitotic cells in spleen, especially at margins of follicles.
	White blood count	450		7,500		
	Polynuclears		40%	12%	88%	
	Mononuclears		60%	88%	12%	

large variety, whereas those coming from the adenoid tissue of the intestines and mesenteric nodes were mainly of the small variety. The significance of this was clearly shown in cat 3, where the white cells coming from the spleen were found to be only half as numerous as in the peripheral circulation and in the inferior mesenteric vein. The autopsy showed the reason for this. The spleen was unchanged and discharged its normal quota of large mononuclears into the blood stream; but the adenoid tissue in the mesentery was enormously hypertrophied and was discharging great numbers of small lymphocytes into the circulation. Microscopic sections showed the efferent vessels of these nodes to be packed with small mononuclears.

One of the most striking evidences of splenic blood formation was that of cat 2, in which 13,000,000 red cells and 137,000 white cells per cubic millimeter were found in the splenic vein as against about two thirds of that number in the peripheral circulation. The reason for this at once became apparent on examination of the spleen itself. There was an enormous hyperplasia of the organ, and the germinal centers on microscopical section showed very active production of large mononuclears. There were furthermore two accessory spleens. The organs elsewhere were normal and the cat seemed in good health.

Another marked instance of the difference between splenic and portal corpuscular content was seen in dog 2, in which the proportions of large and small mononuclears were almost exactly the reverse in the two veins.

Most interesting were the results in rabbit 5, which had been previously inoculated with a rather virulent strain of paratyphoid

bacillus. This strain in rabbits causes a marked leucopenia in the systemic vessels as a constant feature following inoculation. But the remarkable thing was that although this leucopenia had reached such a grade that the heart's blood contained only 450 white blood cells to the cubic centimeter the spleen still kept discharging leucocytes at a rate of 7,500 per cubic centimeter, a perfectly evident effort at compensation for the loss in the general blood stream.

These results all point to the inevitable conclusion that the spleen is a blood-forming organ of prime importance in the animal metabolism. The fact that the organ can be extirpated without causing death or even considerable detriment to the animal organism does not militate against this conclusion. Other organs (hemolymph nodes, bone marrow, and adenoid tissues in general) may assume part of the rôle of the spleen when this is absent, but only the severity of the blood-destroying agent and the individual resistance can determine whether the body can stand the strain when deprived of the spleen. Cases of death from removal of the malarial spleen indicate this strongly. And this same individual variation will doubtless account for the discrepancy in the results obtained by different observers regarding the importance of the spleen in the animal economy.

In closing, I desire to express my gratitude to Dr. William G. MacCallum for his interest in this work.

ON THE IMMUNIZATION OF ANIMALS WITH BACTERIAL PROTEOTOXINS (ANAPHYLATOXINS).*

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The discovery, a few years ago, by Friedberger (1) that powerful poisons were produced when active guinea pig serum was left together with emulsions of various bacteria, has had an important influence upon our conceptions of infectious disease, and, indeed, has furnished cause for reconsideration of the long accepted endotoxin theory of Pfeiffer. It has become at least a reasonable hypothesis that these toxic products of Friedberger, which he has, perhaps somewhat inaptly, named anaphylatoxins,[1] may be the sole factors in the toxemias of such diseases.

Our knowledge of these poisons is, of course, in many features incomplete. Their relation to the actual mechanism of serum anaphylaxis is not positively determined and, since this phase of the subject is quite distinct from the work reported in this paper, we may abstain from discussing it here. In the case of the bacterial anaphylatoxins more particularly, the work of the last few years has opened the important question as to whether or not the bacterial protein actually furnishes the matrix or substrate for the poison. This was the natural assumption of Friedberger and seemed also to follow from the earlier work of Vaughan (2), since there are so many distinct points of similarity between the complement-produced substances of the former and the toxic split products obtained with the aid of purely chemical treatment by the latter. This view is further supported by a number of workers by the apparently autolytic production of such poisons from bacteria in salt solution suspension.

However, the work of Keysser and Wassermann (3), in which anaphylatoxin-like poisons were produced in guinea pig serum when kaolin or barium sulphate was substituted for bacteria, seemed to contradict this conception, and of similar import are certain experiments of Bordet, and the recent work of Jobling and Petersen (4). The last named investigators especially have left little room for doubt that poisons apparently similar to those of Vaughan and

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[1] Although we have long hesitated further to complicate the nomenclature of this involved subject, we believe that much confusion can be avoided by substituting the term proteotoxins for all of the poisons of this class,—viz., poisons produced by the contact of active serum with bacteria, precipitates, kaolin, agar, starch, etc.

of Friedberger (Jobling and Petersen call them serotoxins) can be produced by serum enzymes from the proteins of the serum itself, when these have been exposed by the adsorption of anti-enzymes by the kaolin or by the bacteria.

It is somewhat uncertain, therefore, whether the poison engendered when bacteria and fresh serum are put together at 37.5° C. is a product of bacteriolysis, as at first supposed by Friedberger, or whether it is rather a product of the cleavage of other proteins, a process in which the bacteria play only an indirect part. It is impossible to settle this point at present, although we do not think that the occurrence of one process necessarily excludes the other.

However this may be, it has no direct influence upon the importance of poisons of this description in infectious disease. Whatever the matrix, the fact remains that they are produced when active serum constituents meet with bacteria in suitable quantitative proportions and at body temperature. Their actual occurrence when these elements meet within the animal body has been demonstrated experimentally by Friedberger and Nathan (5). We are, therefore, still justified in attributing to them an important part in bacterial toxemia, in accordance with the theory of Vaughan and the subsequent views of Friedberger.

This being the case, it is of great importance to our general conceptions of immunity to determine whether or not the animal body can develop increased resistance to these poisons. Our own studies were carried on in direct connection with work upon immunity in typhoid fever, a disease in which one of us in a previous paper (6) has attempted to show the great likelihood of participation of the anaphylatoxins.

In spite of a number of investigations bearing directly upon this point, we were unable to find a definite answer to this question, when, incidental to a general study of such poisons, we reviewed the literature.

As early as 1905, V. C. Vaughan, Jr., (7) attacked this problem in connection with the toxic split products obtained by the method of V. C. Vaughan, Sr., from the colon bacillus. His results justified him in concluding that "after the administration of several doses of gradually increasing strength, a point is reached at which the animal is able to withstand the injection of from two to three times the amount which would surely have proven fatal for an untreated control." He interpreted this rather as an evidence of acquired tolerance than as one of immunity in the ordinary sense of the word.

Bessau (8) later studied the same question by a number of different methods, both in relation with serum anaphylaxis and with bacterial anaphylatoxins directly. In one series of experiments he sensitized guinea pigs simultaneously with beef and with horse sera and, after the proper period of incubation, when full susceptibility was developed, he administered sublethal doses of one of these sera. After recovery, animals so treated were found to be less susceptible to reinjection with the other serum than were controls to which the latter serum only was given. These results obviously seem to indicate that the preliminary intoxication with the anaphylactic poison induced a non-specific tolerance.

Though indirectly of much interest, the importance of these experiments for the solution of the problem we are discussing rests upon the acceptation of the identity of the so called anaphylatoxins (proteotoxins) with the toxic sub-

stances involved in serum anaphylaxis,—an assumption which is indeed rendered likely by many observations, but which, after all, is not yet a matter of certainty. Of more direct bearing, therefore, are the other experiments of Bessau in which animals that had recovered from anaphylactic shock (both actively and passively prepared), were subsequently injected with typhoid anaphylatoxin (proteotoxin). Here, too, it was found that the animals receiving the preliminary treatment showed but slight symptoms and recovered from doses of the poison which killed the untreated controls. Bessau concludes from his experiments that anti-anaphylaxis is a condition based entirely upon this acquired and non-specific tolerance. We will discuss this view in our conclusions.

Ritz and Sachs (9) later claim to have observed that guinea pigs were protected against *B. prodigiosus* anaphylatoxin after they had been injected with a sublethal dose of the same substance, 20 to 45 minutes before.

Subsequently Friedberger and Lurà (10) repeated and contradicted the experiments of Bessau. In their conclusions they state that animals treated with sublethal doses of anaphylatoxin are just as susceptible as normal animals if reinjected after 24 hours with anaphylatoxin prepared either from homologous or from heterologous protein. They likewise obtained negative results when they treated serum sensitized animals with anaphylatoxin and subsequently reinjected the protein used for sensitization. The animals so treated remained as sensitive as the controls.

The available literature, though on the whole favoring the occurrence of such acquired tolerance to poisons of this description, is contradictory. Moreover, the few experiments cited by the investigators mentioned above, in which repeated injections of bacterial proteotoxin were given, are not sufficiently extensive to be convincing either affirmatively or negatively. For, as our protocols will show, there is frequently observed a marked normal difference in susceptibility to these substances by guinea pigs of the same weight and age,—a confusing factor which necessitates the study of large series before a conclusion can be reached.

METHODS.

The plan of our experiments was a simple one, aimed solely at determining whether guinea pigs treated with sublethal doses of anaphylatoxin, produced by putting together bacteria and fresh guinea pig serum, could be thereby rendered more resistant to subsequent injections of this poison.

The toxic substance was invariably prepared with typhoid bacilli in the following way: Typhoid bacillus, laboratory strain "J," was grown on agar slants for twenty-four hours and the growth was

taken up with one cubic centimeter of salt solution for each slant. This emulsion was then mixed with fresh guinea pig serum in proportions of one half agar slant to each four cubic centimeters of serum. The mixture was incubated for six hours and then centrifugalized for one to two hours. It should be stated that centrifugalization, however long continued, never completely freed the serum of bacteria. We tried subsequently to accomplish this by the addition of small amounts of inactivated, strongly agglutinating serum to the mixtures before centrifugalization, but even then a few microörganisms invariably remained in suspension.

Filtration through Berkefeld candles removed the bacteria but, strangely enough, also rendered the serum non-toxic. This is an observation which greatly surprised us, but which, as we later found, had also been made by Moreschi and Golgi (11). For these reasons it was not possible to work with an absolutely bacteria-free preparation. However, since our experiments deal with resistance to the acute effects of the poison itself, this fact does not in any way complicate our results.

Both the immunizing doses and the test injections were given intravenously, preliminary experiments with intraperitoneal immunization having been entirely negative. As will be seen, it appeared to us necessary to carry out a very large series of experiments before we ventured to draw conclusions. Every now and then guinea pigs are met with which show unusual susceptibility or unusual resistance to the poisons. One or two such animals in a series tend to upset confidence in the results and necessitate repetition. For these reasons also it was necessary to use almost as many controls as test animals. The controls were always heavier than the test guinea pigs, and controls were made both before and after the test guinea pigs were injected, in order to allow for possible alterations in toxicity during the period of incubation, which often exceeded one half hour.

EXPERIMENTS.

In tabulating the experiments we have purposely omitted recording the preliminary observations by which the toxicity of each particular poison, used for the first or immunizing dose, was determined. In giving the first injections we aimed to employ a dose

TABLE I.

Experiment 1.

An interval of 2 days was allowed to elapse between the first and the second injections.

No. of animal.	First weight, gm.	First dose.	Result.	Second weight, gm.	Second dose.	Result.
1	240	3.0 c.c.	Severe shock	235	3.5 c.c.	Death in 3½ min.
2	230	3.0 c.c.	Moderate shock	230	3.5 c.c.	Death in 2½ min.
3	235	3.0 c.c.	Moderate shock	230	3.4 c.c.	Death in 4 min.
4	225	3.25 c.c.	Moderate shock	220	2.5 c.c.	Death in 5 min.
Controls before experiment						
1				240	2.5 c.c	Severe shock. Recovered.
2				230	3.5 c.c.	Death in 3 min.
Controls after experiment						
1				230	3.5 c.c.	Death in 3½ min.
2				225	3.5 c.c.	Very severe shock. Recovered.

In this experiment there is no evidence of increased resistance in the animals, if tested two days after the first administration of the poison.

TABLE II.

Experiment 2.

An interval of 4 days was allowed to elapse between the first and the second injections.

No. of animal.	First weight, gm.	First dose.	Result.	Second weight, gm.	Second dose.	Result.
1	225	3.25 c.c.	Very severe shock Recovery	225	3.5 c.c.	Death in 3½ min.
2	225	3.25 c.c.	Severe shock	225	3.5 c.c.	Death in 5 min.
3	200	3.25 c.c.	Moderate shock	200	3.5 c.c.	Death in 2½ min.
4	200	3.25 c.c.	Severe shock	200	2.5 c.c.	Death in 2¾ min.
Controls before experiment						
1				235	3.0 c.c.	Severe shock. Recovery.
2				240	3.5 c.c.	Death in 3½ min.
Controls after experiment						
1				225	3.5 c.c.	Death in 4 min.
2				190	3.5 c.c.	Severe shock. Recovery

Here there is no evidence of increased tolerance if reinjection is practised after an interval of 4 days.

TABLE III.

Experiment 3.

An interval of 7 days was allowed to elapse between the first and the second injections.

No. of animal.	First weight, gm.	First dose.	Result.	Second weight, gm.	Second dose.	Result.
1	195	2.0 c.c.	Severe shock	200	3.5 c.c.	No shock.
2	210	2.5 c.c.	Severe shock	205	3.5 c.c.	No shock.
3	210	2.5 c.c.	Slight shock	185	3.0 c.c.	Very slight shock.
4	205	2.0 c.c.	Slight shock	200	3.0 c.c.	No shock.
5	195	2.0 c.c.	Slight shock	195	3.0 c.c.	Moderate shock. Recovery.
6	195	2.0 c.c.	Moderate shock	200	3.5 c.c.	No shock.
Controls before experiment						
1				210	3.0 c.c.	Death in 3½ min.
2				200	2.0 c.c.	Death in 5 min.
Controls after experiment						
1				235	2.5 c.c.	Death in 2¼ min.
2				240	2.5 c.c.	Death in 4 min.

In this experiment we obtained strong evidence that after 7 days a considerable degree of tolerance to the poison was established. The poison here used was exceptionally powerful and killed the heavier controls without exception in doses of 2 to 3 c.c., whereas the test animals, all of them lighter than the controls, lived, some of them showing no shock whatever.

TABLE IV.

Experiment 4.

An interval of 14 days was allowed to elapse between the first and the second injections.

No. of animal.	First weight, gm.	First dose.	Result.	Second weight, gm.	Second dose.	Result
1	220	3.0 c.c.	Severe shock	220	5.0 c.c.	Death in 3 min.
2	260	3.5 c.c.	Severe shock	217	4.0 c.c.	Slight shock. Lived.
3	250	3.5 c.c.	Severe shock	240	4.5 c.c.	Slight shock. Lived.
4	250	3.5 c.c.	Severe shock	230	4.0 c.c.	Slight shock. Lived.
Controls before experiment						
1				255	4.0 c.c.	Death in 2 min.
2				245	3.0 c.c.	Death in 2½ min.
3				235	2.5 c.c.	Death in 4 min.
Controls after experiment						
1				260	4.0 c.c.	Death in 3 min.
2				240	3.0 c.c.	Death in 3½ min.

Again we have evidence of the development of tolerance, as in the preceding experiment.

which would give moderate shock without killing the guinea pig. The controls mentioned in the tables are always those carried out with the poison used for the second injection, by which the resistance of the previously injected guinea pigs was tested.

TABLE V.

Experiment 5.

An interval of 15 days was allowed to elapse between the first and the second injections.

No. of animal.	First weight, gm.	First dose.	Result.	Second weight, gm.	Second dose.	Result.
1	185	2.5 c.c.	Severe shock		4.5 c.c.	Slight shock. Lived.
2	220	3.5 c.c.	Slight shock		4.5 c.c.	No shock. Lived.
3	225	3.0 c.c.	Moderate shock	Not	5.0 c.c.	No shock.
4	180	2.5 c.c.	Slight shock	taken for	4.5 c.c.	No shock.
5	185	2.5 c.c.	Slight shock	these	4.5 c.c.	Death in 4 min.
6	180	2.5 c.c.	Slight shock	seven	4.0 c.c.	No shock.
7	225	4.0 c.c.	Very severe shock	animals	5.0 c.c.	No shock.
Controls before experiment						
1				195	3.5 c.c.	Death in 2 min.
2				255	4.0 c.c.	Severe shock. Lived.
3				300	4.5 c.c.	Severe shock. Lived.
Controls after experiment						
4				235	2.5 c.c.	Death in 2 min.
5				240	2.5 c.c.	Death in 2½ min.
6				240	2.5 c.c.	Death in 3½ min.
7				210	2.5 c.c.	Death in 3 min.

This experiment, while supplying strong evidence in favor of the development of tolerance, illustrates most clearly the irregularity occasionally encountered in the reactions of normal guinea pigs. Note how five of the seven controls died of relatively small doses, in acute shock, whereas two slightly heavier animals, after severe shock, survived larger amounts. It is to be noted in this connection that small differences of weight often mean considerable differences of resistance, indicating probably that the younger guinea pigs are much more susceptible than the older ones, the difference being greater than could be accounted for by mere difference in weight.

The preceding protocols (tables I to V) sufficiently illustrate the methods by which our experiments were done. A complete tabulation of all our experiments, which include over seventy test animals and sixty controls, would needlessly lengthen our paper without materially adding to clearness. For this reason we will briefly summarize the results in tables VI and VII, adding explanatory re-

marks since it was not possible to include the weights of the individual animals and the dosage employed.

TABLE VI.

Resistance to the Poison of Animals Which Had Received One Previous Injection.

Second injection, after 2 to 5 days.		Controls.	
Total No. of animals.	No. of animals surviving.	Total No. of animals.	No. of animals surviving.
12	2	12	6

The dosage here was practically the same. The weight of the control animals averaged from 10 to 15 gm. more than that of the test animals at the second injection.

The results if analyzed in the individual experiments show that before the fifth day, as a rule, the guinea pig is not only more resistant or tolerant to the poison, but is still suffering from the effects of the first injection and rendered thereby even less resistant than normally.

TABLE VII.

Second injection, after 7 to 60 days.		Controls.	
Total No. of animals	No. of animals surviving.	Total No. of animals.	No. of animals surviving.
45	24	35	7

The dosage here was often one and one half to twice as high in the test animals as in the controls. The controls in the individual experiments were always heavier than the test animals.

There can be no question, in these experiments, that a definite degree of increased resistance is present in the animals between the seventh and the sixtieth days. It should be noted that, in analyzing the individual protocols, the most uniformly positive results were obtained between the seventh and the fifteenth days.

We have records of a few animals in which the interval between the immunizing and the first injection exceeded sixty days, but in none of these has there been as consistent or reliable evidence of acquired tolerance as in the cases recorded above, in which the intervals were shorter.

SUMMARY.

Our experiments have shown definitely that guinea pigs, once injected with sublethal doses of bacterial proteotoxins (anaphylatoxins), acquire distinct tolerance to these poisons. The degree to which such resistance or tolerance is developed is never very high, in no case in our experiments exceeding the ability to withstand one and one half to twice the fatal dose of the poisons. During the three or four days immediately following the first injection the animals appear to be slightly less resistant than are normal controls, this depending probably upon the injury done by the administered poison. Tolerance begins to be evident after from four to seven days, seems to be most highly developed in about two weeks, but lasts in a diminishing degree for at least as long as sixty days.

Our experience, in this respect, with the poisons resulting from the contact of active serum and bacteria is similar to that of Vaughan with the toxic protein split products obtained by chemical methods.

The development of increased resistance definitely established, the questions immediately arise: (1) Is this tolerance specific? And (2) can it be passively transferred, with the serum, to a normal animal? We have begun to seek answers for these problems but as yet our data are too meager to permit definite conclusions.

The significance of the existence of higher resistance in animals treated with proteotoxins is far reaching both in connection with anaphylaxis and with immunity in general. We are not inclined to attribute to it as predominant a part in anti-anaphylaxis as is assigned to it by Bessau. For, in the first place, tolerance to the poisons is never developed to a very high degree, and, moreover, it does not become evident until three or four days after the first injection, while anti-anaphylaxis develops almost immediately after shock. However, there seems to us to be strong presumptive evidence that such tolerance to the poisons may play an important and, possibly, a non-specific part in anti-anaphylaxis, the chief underlying and specific cause of this phenomenon being the exhaustion of antibodies, or desensitization in the sense of Besredka.

The relation of such tolerance to the resistance of the animal to bacterial infection is, of course, obvious if we accept the possibility

of the production of such poisons in the injected body and their participation in the production of bacterial toxemia. We hope to throw more light on these relations in another paper dealing with the aggressin-like properties of the proteotoxins.

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EXPERIMENTS ON THE RÔLE OF LYMPHOID TISSUE IN THE RESISTANCE TO EXPERIMENTAL TUBERCULOSIS IN MICE.*

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In the reaction to tuberculous infection the part played by the endothelioid cell or endothelial leucocyte (Mallory) has received much attention in recent years. This is probably due to the early appearance of these cells in the process of formation of the tubercle and because of their well known phagocytic activities.[1] The other conspicuous element in tubercle formation, the lymphoid cell, probably because of its later appearance and its lack of phagocytic power, has been almost entirely neglected. The small round cell infiltration about the tubercle has indeed been regarded by most observers as of very secondary importance and is often referred to as resulting probably from tissue destruction.

In spite of the lack of interest among pathologists concerning the rôle of the lymphocyte in the tuberculous process, attention has been called by a number of clinicians to the prognostic importance of the lymphocytes in the circulating blood.[2] These observers have noted that in rapidly fatal miliary tuberculosis the lymphocytes fall, often to below 10 per cent. of the circulating white cells, whereas in patients with early, healed, or healing tuberculous lesions these cells are increased, sometimes forming more than 50 per cent. of the total leucocytes.[3] Moreover, it is well known that in acute rapidly fatal

* Received for publication, August 18, 1914.

[1] For the literature see Goldmann, E. E., Neue Untersuchungen über die äussere und innere Sekretion des gesunden und kranken Organismus im Lichte der "vitalen Färbung," Tübingen, 1912, 52; and Evans, H. M., Bowman, F. B., and Winternitz, M. C., *Jour. Exper. Med.*, 1914, xix, 283.

[2] For the literature, see Brecke, A., in Brauer, L., Schröder, G., and Blumenfeld, F., Handbuch für Tuberkulose, Leipzig, 1914, i, 581.

[3] Wack, P., *Deutsch. Arch. f. klin. Med.*, 1914, cxv, 596.

miliary tuberculosis relatively few lymphocytes occur in the individual tubercles, while in the subacute form where a higher resistance of the individual may be assumed, the tubercles contain large numbers of lymphoid cells. These facts have a close analogy in the reaction of the polynuclear leucocytes in certain infections against which they are presumed to form the chief resisting factor.

LYMPHOCYTES AS FACTORS OF RESISTANCE.

Until recently, the round cell infiltration occurring about slowly growing or healing cancer, about cancer grafts in immunized animals, about failing tissue grafts in unsuitable or resistant animals, has been assigned, as in tuberculosis, a secondary rôle. The lymphoid elements here, too, are supposed to be present as a result of tissue disintegration. However, it has been shown that in the case of tissue grafts, the lymphocytes in all probability are the chief agents in causing the destruction of the introduced tissue. The chick embryo normally has no resistance against the growth of implanted tissues from a foreign species, and likewise shows a total absence of the round cell infiltration about the graft.[4] When, however, the chick embryo is provided with a graft of adult chicken lymphoid tissue, it becomes as resistant as the adult to the growth of implanted tissue from a foreign species, and, like the adult, shows an intense infiltration of small round cells about the foreign graft.[5] Furthermore, if the lymphoid system of an adult animal is depleted by means of X-ray, the animal loses its power of resistance to heterologous tissue and an implanted tissue from a foreign species will then grow readily. It is significant that in such animals there is a total absence of the round cell infiltration about the edges of the graft always present in resistant untreated animals.[6]

In the light of these results it seemed probable that the lymphoid cell might play a more important rôle in the resistance to certain infections than had previously been supposed. The fact of its presence in the reaction to tuberculous infections, its variation in the blood with the condition of the tuberculous individuals, and the recent ex-

[4] Murphy, Jas. B., *Jour. Exper. Med.*, 1913, xvii, 482.

[5] Murphy, Jas. B., *idem*, 1914, xix, 513.

[6] Murphy, Jas. B., *Jour. Am. Med. Assn.*, 1914, lxii, 1459.

periments of Lewis and Margot[7] suggested the advisability of investigating the part played by the lymphocyte in the resistance to tuberculosis. Lewis and Margot made the observation that rats and mice experimentally infected with tuberculosis developed large spleens. Splenectomized animals, however, lived longer after inoculation than normal animals. This anomalous result seemed difficult to explain. It is interesting to note in this connection that the inoculations were never made in less than two weeks after the splenectomy and were usually done in the third week. At this period the lymph glands and the lymphoid tissue of the body have shown considerable hypertrophy and many animals show a great increase over normal in the circulating lymphocytes.[8]

THE EFFECT OF X-RAY ON LYMPHOID TISSUE.

Heineke has shown that X-ray has an almost specific and immediate destructive action on the lymphoid system, and in small doses seems to have little, if any, effect on other cells of the body.[9] These results have been adequately confirmed by other observers. We have found that by carefully regulated doses of X-ray, repeated at intervals, a gradual atrophy of the lymphoid tissue may be accomplished without any appreciable effect on the other tissues, or on the general health of the animal.

TUBERCULOUS INFECTIONS IN X-RAYED MICE.

This specific effect of X-ray on the lymphoid tissue offers an excellent experimental method for testing the value of the lymphocyte in various conditions. Animals whose lymphoid tissue has been destroyed should be highly susceptible to those infections against which the lymphocytes play a part in the defense, while their resistance to those infections met by the polymorphonuclear leucocytes should be unaffected. With this idea in view the following experiments were planned.

[7] Lewis, P. A., and Margot, A. G., *Jour. Exper. Med.*, 1914, xix, 187.

[8] Dr. Linda B. Lange has made a series of differential counts on mice before and at intervals after splenectomy. These will probably be reported later.

[9] Heineke, H., *Mitt. a. d. Grenzgeb. d. Med. u. Chir.*, 1905, xiv, 21.

Experiment 1.—Fifty mice of about the same age and size were selected. These were divided into lots of ten each and subjected to the following treatment.

A. Ten mice were splenectomized and then given a daily 5-minute exposure to X-ray for 2 weeks. Average dosage less than 1/4 unit (Holzknecht scale), average penetration No. 6, milliamperes 3–4. Spark gap 1 to 1½ inches.

B. Ten normal mice were given daily exposures to X-ray for 2 weeks in the same dosage as group A.

C. Ten mice were splenectomized on the date of inoculation 4 to 9 hours before this procedure.

D. Ten mice were splenectomized 8 to 10 days before the date of inoculation.

E. Ten normal animals served as controls.

All the animals were in good condition at the time of inoculation.

They were divided into small groups in order to prevent the spread of epidemics if any should develop, and were then inoculated with an emulsion of a 6 weeks' glycerin veal bouillon culture of bovine tubercle bacilli, each animal receiving a dose of 1 mg. of dry tubercle bacilli in 0.8 c.c. normal salt solution.[10] As control to the X-ray effect numerous mice have been given the same or larger doses and have shown no bad effects while under observation for several weeks afterward.

TABLE I.

Animals.	Average time of survival.	Percentage of animals with tubercle bacilli in various organs.					
		Exudate.	Spleen.	Liver.	Heart's blood.	Lungs.	Kidney.
Group A	7.3 dys.	100		100	40	80	100
Group B	7.0 dys.	100	100	100	60	80	100
Group C	9.1 dys.	100		100	50	80	100
Group D	19.7 dys.	100		100	90	100	100
Group E	18.5 dys.	100	100	100	80	70	100

Group A = 10 splenectomized mice given 14 daily exposures to X-ray. Group B = 10 normal mice given 14 daily exposures to X-ray. Group C = 10 mice splenectomized a few hours before inoculation. Group D = 10 mice splenectomized 8 to 10 days before inoculation. Group E = 10 normal mice as controls.

Table I shows the average number of days the various groups of animals lived and the percentage of animals showing tubercle bacilli in the blood, peritoneal exudate, and the various organs. The two X-ray groups, A and B, lived on an average about seven days after the inoculation, and group C, splenectomized a short time before inoculation, averaged only about nine days. Group D, the ani-

[10]This culture was kindly provided by Dr. Paul A. Lewis of the Henry Phipps Institute. It is termed by him Bovine C. The organisms were dried by pressing between filter papers, weighed, and then made into an emulsion by long grinding in normal salt solution.

mals splenectomized about ten days previous to the inoculation, averaged 19.7 days which is a little more than a day longer than the normal group E, which survived 18.5 days as an average. The widespread distribution and great number of organisms leave little doubt that the tubercle bacilli were the cause of death. To rule out epidemics of mouse typhoid, cultures were always taken from the heart's blood and the character of any organisms obtained was studied.

Experiment 2.—This experiment confirms and adds a further control to experiment 1. The groups of mice used were as follows:

A. Eight mice were splenectomized and given 12 daily exposures to X-ray in the same dosage as in experiment 1.

B. Nine normal mice were given the same X-ray exposures as those in group A.

C. Sixteen small normal mice were given 17 daily exposures of X-ray of the same intensity as those in groups A and B. The exposures were discontinued 4 weeks before the inoculations were made.

D. Ten mice were splenectomized 3½ weeks before inoculation.

E. Ten normal mice.

All these animals except 8 of group C were given 2 mg. of a 9 weeks' old culture of bovine tubercle bacilli of the same strain as that used in the first experiment. These animals were isolated in individual glass jars so as to prevent the spread of epidemics, should any develop.

TABLE II.

Animals.	Average time of survival.	Percentage of animals with tubercle bacilli in various organs.					
		Exudate.	Spleen.	Liver.	Heart's blood.	Lungs.	Kidney.
Group A	8.4 dys.	100		80	20	40	40
Group B	9.7 dys.	100	100	66	11	44	55
Group C	7.1 dys.	100	88	71	42	57	100
Group D	26.0 dys.	75		87	25	37	62
Group E	23.3 dys.	100	100	80	10	90	70

Group A = 8 mice splenectomized and given 12 daily exposures to X-ray. Group B = 9 normal mice given 12 daily exposures to X-ray. Group C = 16 mice given 17 daily exposures to X-ray; 8 of them were inoculated 4 weeks after X-ray was discontinued. Group D = 10 mice splenectomized 3½ weeks before inoculation. Group E = 10 normal mice.

Table II shows the death rate and distribution of the organisms. The age of the culture explains the longer survival of the animals even with twice the dose used in the first experiment. Groups A and

B, the splenectomized X-rayed and the normal X-rayed animals, lived an average of 8.4 and 9.7 days, respectively, after inoculation. Of the sixteen mice in Group C, the life of the eight which were inoculated averaged only 7.1 days after inoculation. Although these animals had had a month to regenerate their lymphoid tissue, they still showed a completely depressed resistance. As a matter of fact, the autopsies showed the spleens and lymph glands to be atrophic, with little sign of regeneration. The early death in these animals compared with the other X-rayed animals may perhaps be due to the fact that they were much smaller mice and had been given more frequent exposures to X-ray. Eight mice of this lot which were not inoculated but kept as a control to X-ray effect are still living and in perfect condition almost three months after X-ray treatment. The mice splenectomized three and a half weeks before inoculation, in agreement with the results of Lewis and Margot, lived longer than the normal animals.

The experiments were planned with the idea of testing the resistance of the animals to tuberculosis when the amount of the lymphoid tissue was varied. The X-rayed animals are at the bottom of the scale, having a system greatly depleted, and they were the first to die of the disease. Next come those splenectomized shortly before the inoculation; these may be considered as having a reduced amount of lymphoid tissue. The animals inoculated eight to ten days after splenectomy, having an active proliferation of the lymphoid cells in the glands and elsewhere, at this stage apparently have about the same resistance as the normal animals. The animals, however, splenectomized three or more weeks before inoculation outlive the normal animals by a number of days and probably represent an increased activity of the defensive agents.

DISCUSSION.

The question naturally arises: Do these treatments cause variations in factors other than the lymphocyte, which might play a part in the resistance to tuberculosis? X-ray in the amount used in these experiments does not affect the general health of the animal. The polymorphonuclear leucocytes are not decreased in number and may be increased. The X-rayed animals have a normal resistance to

certain infecting agents against which these cells form the defense and in some cases they may even have an increased resistance. The circulating large mononuclear cells are not appreciably affected. As evidence that the endothelioid cells are not destroyed, great numbers of these are found in the spleen and lymph glands after X-ray treatment, actively phagocyting the remains of the lymphocytes.[11]

The well known association of the lymphocytes with tuberculous lesions tends to support the conclusions indicated by these experiments. In the acute miliary type of the disease where it may be supposed that little resistance is being offered, the lymphocytes are relatively few in the tubercle, while in the subacute miliary tuberculosis these cells occur in masses about the lesion. As has already been mentioned, the lymphocytes in the blood fall in the rapidly advancing cases, while individuals with localized and well controlled lesions will show a marked increase in the circulating lymphocytes. It would seem, therefore, that these facts taken in conjunction with our experiments strongly suggest that the lymphocyte plays an important rôle in the animal's resistance to tuberculosis.

SUMMARY.

Mice either normal or splenectomized after exposure to X-ray are markedly more susceptible to bovine tuberculosis than are normal animals. Animals splenectomized a short time prior to inoculation are also more susceptible than normal, while those splenectomized eight to ten days before inoculation have about the same resistance as normal. The mice splenectomized three to four weeks before inoculation have a resistance increased over the normal, as has already been shown by Lewis and Margot. As X-ray in the doses used apparently affects only the lymphoid tissue and as the hypertrophy of the remaining lymphoid tissue after splenectomy is so rapid that the circulating lymphocytes may be much above the normal by the third week, it is concluded that this evidence, taken with the well known association of the lymphocytes with tuberculous lesions, points strongly to the lymphocyte as an important agent in the defensive mechanism against tuberculosis.

[11] Heineke, H., *loc. cit.* See illustrations.

ON THE CAUSE OF THE LOCALIZATION OF SECONDARY TUMORS AT POINTS OF INJURY.*

By F. S. JONES, V.M.D., and PEYTON ROUS, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

PLATES 18 TO 20.

The localization of secondary tumors at points of injury has been so often remarked upon that it is unnecessary to cite specific instances. The cause for the phenomenon is unknown. Lubarsch[1] has shown that mouse tumors may be made to localize secondarily in the liver, about splinters implanted in this organ, but he did not attempt to explain the finding.

There are a number of observations which make it evident that the development of tumor metastases from tumor emboli is conditional upon a special set of circumstances. Schmidt[2] has shown that a large proportion of tumor cells cast off into the blood stream may die without giving rise to metastases. He found that the pulmonary arterioles of patients with visceral cancer often contain many tumor emboli that are dead or dying. The fact has been repeatedly noted that rats and mice inoculated intravenously with tumor fragments seldom develop growths in the lungs despite the fact that the same material causes tumors when implanted subcutaneously. So too in these animals the intraperitoneal inoculation of active tumor material yields comparatively few takes.

It has seemed to us possible to study some of the factors concerned in the secondary localization of tumors by means of experiments involving tumor localization on the lining of the peritoneal cavity. According to Schmidt, the intima of the blood vessels constitutes the essential barrier to invasion of the tissue by the cells of tumor emboli. The peritoneal lining presents much the same structural

* Received for publication, August 1, 1914.

[1] Lubarsch, O., *Med. Klinik*, 1912, viii, 1651.

[2] Schmidt, M. B., Die Verbreitungswege der Karzinome und die Beziehung generalisierter Sarkome zu den leukämischen Neubildungen, Jena, 1903.

features as the intima of the blood vessels, namely, a single layer of flattened cells covering a connective tissue. When tumor fragments are injected into the blood stream they are often surrounded by a thrombus and furthermore are so widely scattered that their fate is difficult to follow. Both these difficulties are avoided by the use of the peritoneal cavity.

The first question to present itself is that of the nature of the forces which prevent, or at least hinder, the take of tumor fragments inoculated intraperitoneally. Is there an immunity reaction in which the fluids of the cavity are concerned, or merely a resistance offered by an intact serosa? To test the point we have injured the peritoneum of mice by mechanical means, afterwards inoculating a tumor. For the purpose finely ground diatomaceous earth (*Kieselguhr*) has been used and an adenocarcinoma known in our laboratory as Mouse Tumor 33. Mouse Tumor 33 grows in discrete masses, seldom infiltrating or becoming diffuse (figure 1). Because of this it was especially suitable for the work.

Experiment 1.—Fifteen mice were injected intraperitoneally with 0.25 c.c. of finely ground, sterile *Kieselguhr* suspended in Ringer's solution, and three days later with 0.1 c.c. of a suspension of very small fragments of mouse carcinoma in Ringer's solution. Control animals were injected with the tumor suspension only.

At the end of two weeks nine of the mice receiving both *Kieselguhr* and carcinoma remained. They were killed and carefully autopsied. Seven had tumors in the subcutaneous tissue and on the peritoneum where the injecting needle had been thrust through. In all of these, nodules were found scattered throughout the peritoneal cavity, and in several the liver, spleen, and kidneys were involved. The remaining two animals were negative.

Eight controls survived the two weeks. Six had tumors along the track of the inoculating needle similar to those in the experimental animals, but in only one had dissemination taken place in the peritoneal cavity.

This experiment and others similar show that an acute injury to the peritoneum, mechanically caused, renders it more suitable for the lodgment and growth of mouse tumor. Microscopic examination of the nodules on the parietal peritoneum, in the mesentery, and on the surface of the liver and spleen of the experimental animals has revealed an interesting condition. The particles of *Kieselguhr* are not distributed evenly but lie in aggregates here and there in the midst of a layer of newly formed and very cellular connective tissue

covered with endothelium. The tumors are in general definitely localized to these areas (figure 2). Many small discrete clumps of neoplastic cells are to be noted lying embedded in the reactive tissue and covered with endothelium. In the case of the larger, more diffuse tumors the association with the *Kieselguhr* is also evident (figure 3).

Although the results are clear cut they do not enable one to conclude whether it is damage to the connective tissue or to the endothelium that renders the peritoneal surface susceptible. For the inoculations were made at a time when the *Kieselguhr* had but just cut its way through the endothelium. In a later series of experiments two weeks were allowed to elapse between the *Kieselguhr* inoculation and the injection of the tumor, in order that the endothelium might have opportunity for complete repair. Sections show that after this time the *Kieselguhr* is enclosed in small discrete accumulations of quiescent connective tissue, completely covered by endothelium.

Experiment 2.—Ten mice were injected intraperitoneally with 0.25 c.c. of finely ground *Kieselguhr* suspended in Ringer's fluid, followed two weeks later by 0.1 c.c. of a suspension of mouse carcinoma. Ten control animals received the tumor suspension only.

Five of the mice that received the *Kieselguhr* and carcinoma were alive two weeks after the injection of the latter. They were killed and examined at this time. In four, tumors had developed along the track of the injecting needle while in the remaining one the tumor had failed to take. In all four susceptible animals tumor nodules were found throughout the peritoneal cavity.

Autopsy of the seven surviving controls revealed the following: Four had growths along the track of the needle and in three the tumor had failed to take. In one of the susceptible animals a nodule was present in the mesentery directly opposite the point of injection. In the others the peritoneal lining was normal.

This experiment and others of the same sort show that an injured peritoneal lining remains favorable to tumor implantations after the endothelium has repaired itself completely. Experiments in which lycopodium spores were used as the foreign body have given identical results. Unlike the *Kieselguhr* the spores do not penetrate but are rapidly surrounded by endothelial cells and later encapsulated by connective tissue (Marchand). As a rule quite a number of them are found lying together in a web of newly formed connective tissue. The little nodules so composed are covered with endothelium (figure 4). They offer a most favorable locus for tumor im-

plantation (figure 5). We feel justified in concluding that it is the derangement of the connective tissue, rather than of the endothelium which renders an injured peritoneal lining favorable to the lodgment and growth of tumor fragments.

The damage caused by *Kieselguhr* or by lycopodium is punctate in character, but it is wide-spread and might conceivably alter the ability of the peritoneum to elaborate immune substances, or to form the medium of their passage. It has seemed necessary, therefore, to perform experiments involving a relatively insignificant and sharply localized damage to the peritoneum. One or several small, sterile glass rods, rounded at the ends, were introduced into the peritoneal cavity of mice through a trocar and followed later by a tumor suspension.

Experiment 3.—Two or three glass rods about 1 mm. in diameter and 8 mm. long were introduced into the peritoneal cavity of each of ten mice. Two weeks later the animals were inoculated intraperitoneally with 0.1 c.c. of a fine suspension of mouse carcinoma. Ten control animals also received the tumor material at this time.

Six of the experimental animals were alive after two weeks. Four had a tumor in the track of the inoculating needle. In all four, intraperitoneal tumors were found situated next to the glass rods and there only (figure 6). The remaining two mice were negative.

Three of the controls survived two weeks. Two had tumors in the injection track and the other was negative. In one of the susceptible animals a few tiny, discrete tumor nodules were found on the mesentery.

The injury caused by a smooth, glass rod where it lies in contact with the peritoneum renders this latter favorable to tumor implantation. An alternative explanation, that the localization of the neoplasm was due to an accumulation of tumor fragments in a dead space about the rods, fails, because there was no dead space, the rods being closely enveloped in mesentery.

Evidently the resistance manifested by a healthy peritoneum to the lodgment and growth of tumor fragments is not due to a general immunity reaction, but is referable to the physical characters of the lining membrane.

In the light of our results, one may ask whether the factor of injury may not play a part, heretofore unrecognized, in the peritoneal dissemination of certain visceral tumors of human beings. It is true that some growths are so malignant that fragments sown on an in-

tact serosa can successfully lodge and grow. This has been noted of certain rat and mouse tumors as well. But at the other end of the scale there are visceral growths which fail to localize on the peritoneal lining, although fragments of them must be distributed to it. With tumors of intermediate malignancy may it not be that the first fragments that are cast off die, and, causing inflammation, render the peritoneal lining more susceptible for future implants? To test the point mice were inoculated intraperitoneally with bits of killed tumor and later with particles of the living growth.

Experiment 4.—Fifteen mice were injected intraperitoneally with 1.0 c.c. of coarse particles of killed mouse carcinoma suspended in Ringer's fluid. The suspension had been heated in the water bath at 55° C. for 15 minutes, a temperature sufficient to kill the cells of the tumor. Three days later each animal received a second inoculation of 0.06 c.c. of a suspension of living tumor fragments. Fifteen control mice were also inoculated at this time.

Seven of the experimental animals were alive two weeks later. In all, tumors had developed in the inoculation track. In six, there were nodules throughout the abdominal cavity. In the remaining animal two discrete nodules were found in the mesentery.

Ten controls survived. Four were completely negative as regards tumor. Six showed tumors in the injection track. Three of these had one or two tiny, sharply circumscribed growths in the mesentery, and a fourth showed many disseminated growths. In the other two the peritoneal lining was healthy looking.

Jobling[3] has shown that the intraperitoneal injection of a suspension of rat carcinoma killed by heat may increase the susceptibility of animals for later subcutaneous implantations of the same tumor. The results of the present experiment might be referred to a similar hypersusceptibility, were it not that in other of our experiments with identical intraperitoneal findings there is no evidence for this, tumors developing along the track of the injecting needle in about the same proportion of control animals and those injected with the killed suspension. Furthermore, special tests have shown that killed suspensions of Mouse Tumor 33 do not induce hypersusceptibility.

Since dead tumor fragments in contact with the peritoneal lining render this latter more suitable for the lodgment and growth of tumor cells, it seems probable that the peritoneal dissemination of some human tumors may indeed come about through the death of the first tumor fragments cast off, and the reaction thus caused.

[3] Jobling, J. W., *Monographs of The Rockefeller Institute for Medical Research*, 1910, No. 1, 52.

In what way does an injury to the peritoneal lining, or, more precisely, to the subendothelial connective tissue favor tumor localization and growth? The observations of Schmidt,[4] already mentioned, offer a suggestion. Schmidt found that tumor cells which had lodged in the pulmonary arterioles were unable to penetrate the vascular endothelium directly, although they might proliferate and ramify within the lumen of the vessel. Whether they ultimately invaded the surrounding tissue depended upon whether they were supplied with a supporting stroma by the subendothelial connective tissue. Now, in the case of the peritoneal lining we have found that the reactive changes caused by an injury to the subendothelial connective tissue greatly favor the lodgment and growth of bits of tumor. It seems possible that the stroma for a tumor fragment might be elaborated with especial ease by a connective tissue in course of proliferation as the result of an injury. As bearing on the point, we have compared the growth *in vitro* of connective tissue reacting to the presence of a foreign body with the growth of normal connective tissue from the same region. Implantations were made into chicken plasma of bits of tissue from about glass rods embedded for various periods in the breast muscle of fowls. Fowls were chosen because they are extremely resistant to local infection and because their plasma can be readily handled.

Experiment 5.—Eight sterile, smooth glass rods about 1.5 by 8 mm. were inserted on successive days by means of a trocar into the pectoral muscles of each of three fowls. The rods were marked for identification. When they had been in place for 1, 2, 3, 4, 6, 8, 10, and 12 days, respectively, the fowls were killed and many small pieces of tissue from about the rods were implanted in chicken plasma and incubated at 41° C. No infection had occurred. Pieces of normal connective tissue, of fascia, and of muscle from near by were also implanted and incubated. The results were striking. No growth occurred from the control fragments or from those about rods that had been in place only one day. There was marked and very prompt emigration of large, rounded ameboid cells from the fragments that had been next to the rods for two or three days. In a few instances there was a definite growth of fibroblasts as well. The pieces removed from about rods that had been in place 4, 5, 6, and 8 days showed a profuse connective tissue growth which began after only a few hours of incubation. In fact, the rapidity and amount of this growth compared very favorably with that of some sarcomata and of embryonic tissue. By the twelfth day the tissue encapsulating the rods had become quiescent and little growth was obtained from it.

[4] Schmidt, M. B., *loc. cit.*

The experiment shows conclusively that connective tissue reacting to the injury caused by the presence of a foreign body has a proliferative energy greater than the normal. Furthermore, its growth in plasma takes place without that latent period which Carrel has described for normal adult connective tissue.[5] The rounded cells that emigrated from the tissue which had been two and three days in contact with the glass rods were doubtless wandering cells attracted by the foreign body; and the true growth that took place obviously came about by the proliferation of the many fibroblasts present in the reactive tissue. From such results it seems highly probable that connective tissue, reacting to an injury, is in a condition to elaborate the stroma for a tumor more rapidly and abundantly than normal tissue.

Altogether, the findings seem to us to indicate that the secondary localization of tumors at points of injury is referable to the presence at such points of a very cellular connective tissue which may come more readily than the normal to the support and nourishment of the tumor cells. A number of facts in the literature may be taken to support this view. To mention only two of them, Loeb and Sweek[6] have described epitheliomata of which the sluggish course was apparently referable to the resistance offered by an inert connective tissue; and Levin[7] has shown that the Flexner-Jobling rat tumor, inoculated into the normal testicle of rats and into testicles previously injected with *Scharlach R* and ether water, will grow only in the latter. Levin ascribes this finding to some chemical influence inducing a "precancerous state" in the testicle. It would seem more likely that it is referable to the presence of a highly labile connective tissue capable of immediate and active proliferation in support of the tumor. The rapid spread of tumor tissue in a wound is explicable on the same basis.

SUMMARY.

The cause of the frequent localization of secondary tumors at points of injury is not known. Our work deals with this problem.

[5] Loeb has found that regenerating kidney grows better than the healthy organ *in vitro* (Loeb, L., *Anat. Rec.*, 1912, vi, 109).

[6] Loeb, L., and Sweek, W. O., *Jour. Med. Research*, 1913, xxviii, 235.

[7] Levin, I., *Jour. Exper. Med.*, 1912, xv, 163.

For the experiments the peritoneal cavity has been employed as offering relatively uncomplicated conditions, and the fate of mouse tumor brought into contact with a peritoneal lining injured in various ways has been studied.

The injection of a suspension of mouse tumor into a healthy peritoneal cavity has little success as a rule compared with a similar injection into the subcutaneous tissue. We have found that the resistance of the peritoneal lining thus indicated can be largely if not completely abolished by the preliminary injection of a mechanical irritant (*Kieselguhr,* lycopodium). That the change thus brought about is independent of general immunity phenomena is shown by the fact that a local injury renders susceptible the part of the peritoneum immediately affected and that part only. Special tests show that the factor important in rendering the peritoneum more susceptible is the injury to the subendothelial connective tissue. Susceptibility persists after the endothelium has regenerated over the reacting connective tissue.

Schmidt has found that the cells of tumor emboli in the pulmonary arterioles are able to penetrate the endothelium of the vessel only after they have been provided with a stroma from the subendothelial connective tissue. Our findings are easily explained on the basis thus suggested. A connective tissue highly cellular and perhaps still proliferating as the result of injury may well elaborate the stroma for a tumor more rapidly than normal connective tissue. Tests of growth *in vitro* support this idea. Connective tissue reacting to an injury grows profusely and almost immediately when incubated in plasma, whereas normal tissue from the same region shows usually no growth whatever.

Dead tumor fragments in contact with the peritoneum cause a change favorable to the lodgment and growth of later tumor fragments. It seems not improbable that the peritoneal dissemination of certain human neoplasms may be accomplished indirectly through the death of the first tumor fragments cast off.

Our observations have been purposely confined to the effects of injury on the peritoneal lining ; but they seem to afford the basis for a generalization. The secondary localization of tumors at points of injury may be attributed with good reason to the presence at such

points of an active connective tissue capable of elaborating a stroma rapidly and abundantly. For it is the proliferation of the subendothelial connective tissue to form a supporting stroma that determines the fate of free tumor cells, whether these lie on the peritoneum or within a vessel.

EXPLANATION OF PLATES.

Plate 18.

Fig. 1. A nodule of Mouse Tumor 33 on the serous coat of the intestine, showing the discrete, uninvasive character of the growth. The nodule is largely necrotic.

Fig. 2. A portion of the parietal peritoneum and abdominal muscle of a mouse receiving an injection of *Kieselguhr* and three days later one of mouse tumor. The animal was killed two weeks after the second injection. On the peritoneal surface are two nodules of reactive tissue containing *Kieselguhr* and a third such nodule in which the tumor has localized.

Plate 19.

Fig. 3. A higher magnification of a portion of the tumor nodule shown in the preceding figure. The *Kieselguhr* is indicated by the arrows.

Fig. 4. Lycopodium spores on the surface of the spleen. They lie grouped together, are embedded in connective tissue, and covered by endothelium.

Plate 20.

Fig. 5. Portion of a tumor associated with the reactive tissue about lycopodium spores. To be compared with figure 4. Part of the abdominal muscle is shown.

Fig. 6. Viscera of a mouse receiving an intraperitoneal injection of tumor fragments two weeks after the introduction into the peritoneal cavity of three small glass rods. There are discrete tumors (*a* and *b*) in the vicinity of the rods but none elsewhere.

ON THE GREATER SUSCEPTIBILITY OF AN ALIEN VARIETY OF HOST TO AN AVIAN TUMOR.*

By PEYTON ROUS, M.D., and LINDA B. LANGE, M.D.

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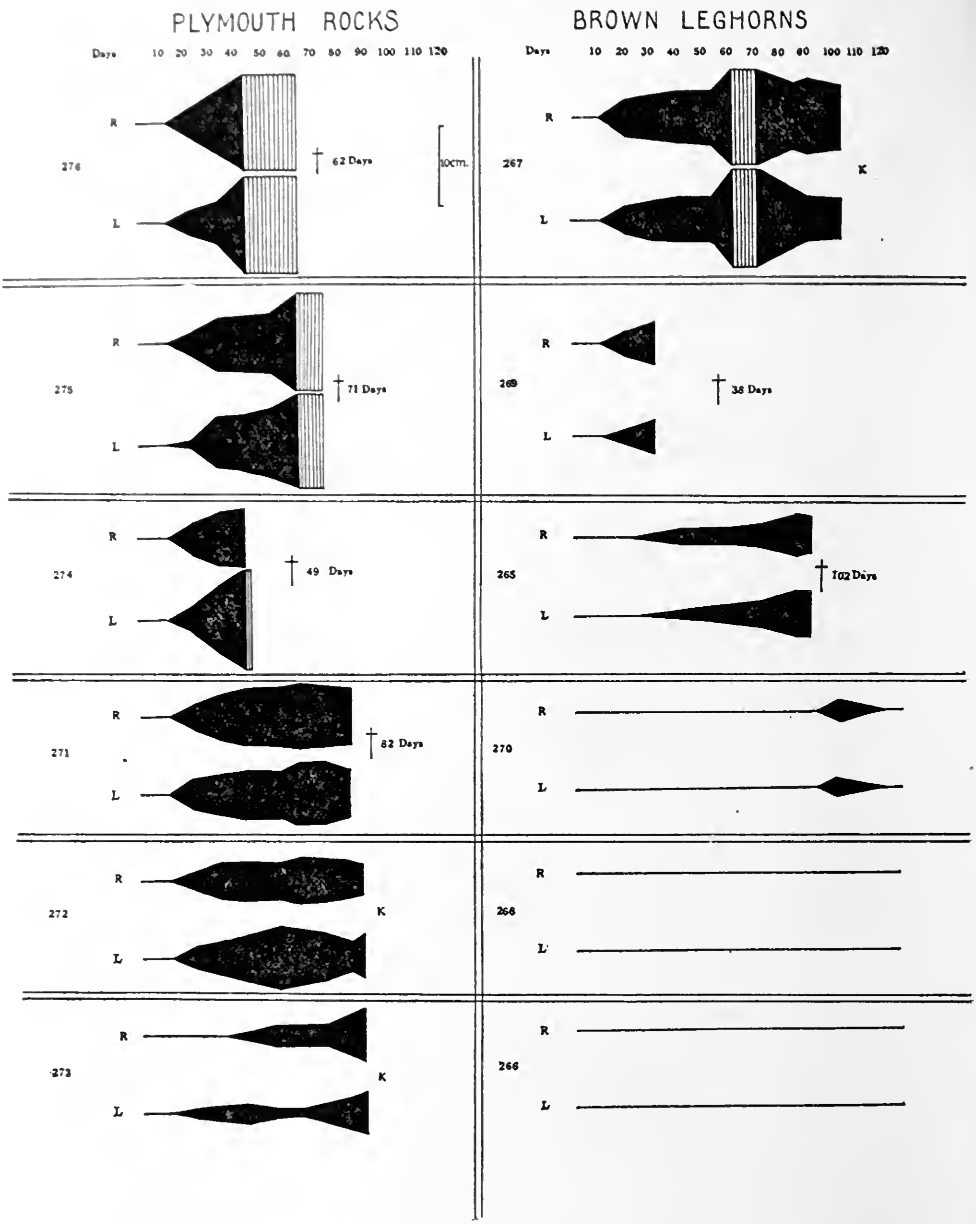
It has been the general experience of workers with transplantable tumors that they are most readily propagated in animals of the variety in which they appear as spontaneous growths (Jensen, Loeb). Some tumors are so specific in their demands as to grow only in individuals from certain sources and not in others of the same race. A spindle-celled sarcoma of the fowl studied in our laboratory was successfully transferred at first only to blood relations of the original host. In fowls of the same variety, but unrelated, it did not grow. The purpose of the present paper is to record an instance in which the behavior of a tumor was against the rule, its transplantation taking place more readily to hosts of an alien variety. Such instances are so rare as to deserve special report.

The growth in question, a tumor of the fowl (Chicken Tumor XVIII), has already been described at length.[1] It is a spindle-celled sarcoma curiously rifted with blood sinuses, and showing a tendency to metastasize to the voluntary muscles. It originated in a brown Leghorn hen, and was transferred to two successive series of such hosts, in which it grew slowly but with some increase in the percentage of takes. Of the third series of fowls inoculated several were barred Plymouth Rocks, and in these the tumor grew with especial rapidity. On subsequent inoculation to other barred Plymouth Rocks its growth was still more rapid. The findings seemed attributable either to an enhanced malignancy consequent on passage and independent of the host's variety, or to some special susceptibility of the Plymouth Rock breed. To test the matter a number of comparative inoculations were made.

* Received for publication, August 1, 1914.

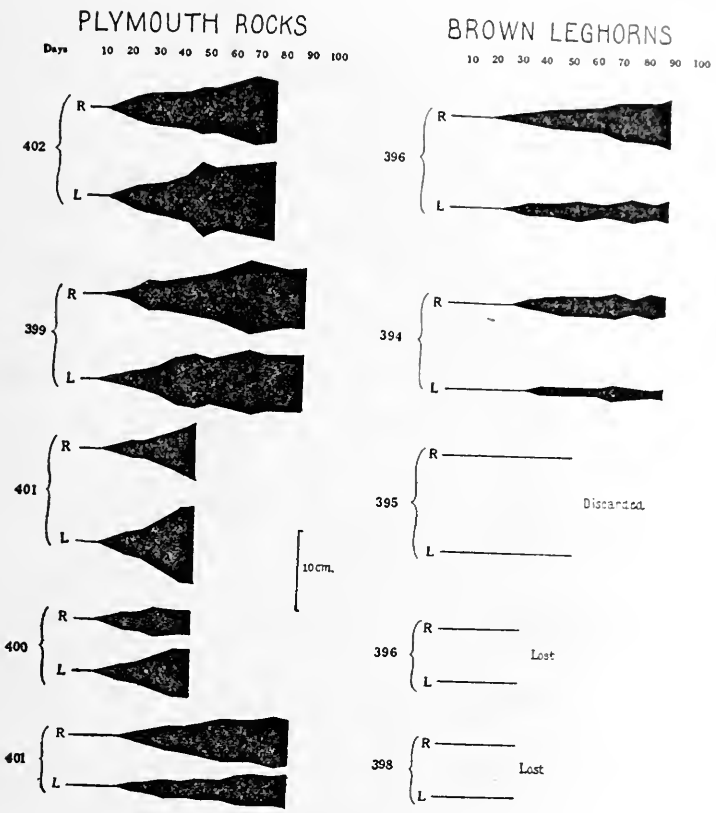
[1] Rous, P., and Lange, L. B., *Jour. Exper. Med.,* 1913, xviii, 651.



TEXT-FIG. 1. Results of the inoculation into Plymouth Rock and brown Leghorn fowls of bits of the rifted sarcoma taken from a Plymouth Rock. Two inoculations were made into each fowl. The time of appearance and rate of growth of the tumors are shown by diagrammatic outlines of which the width indicates the diameter of the tumor, and the length the period of growth. A hatched outline indicates that the tumor had filled the whole breast and could not be accurately measured. R and L = tumors of right and left breast. K = killed. The changes in size of the tumors appear abrupt, but this is because the measurements were taken at considerable intervals.

Experiment 1.—Small bits of fresh tumor tissue, of approximately equal size, from a barred Plymouth Rock fowl were implanted through a trocar, one in each breast of six brown Leghorn adults and six Plymouth Rocks. The results of the transplantation are shown in text-figure 1.

Experiment 2.—The tumor material was taken from a brown Leghorn inoculated in the preceding experiment. Implantations were made as before into each breast of five Plymouth Rock and five brown Leghorn fowls (text-figure 2).



TEXT-FIG. 2. Results of the inoculation into Plymouth Rock and brown Leghorn fowls of bits of the rifted sarcoma from a brown Leghorn fowl of the experiment recorded in text-figure 1.

Text-figure 1 shows that the sarcoma taken from a Plymouth Rock fowl and inoculated into Plymouth Rocks and brown Leghorns appeared in a smaller percentage of the latter and after a longer interval, and grew less well. This was also the case when

the material was derived from a brown Leghorn fowl (text-figure 2). Further observations have confirmed these findings. It may be urged that in the experiments figured, the results were due to an adaptive change in the tumor consequent upon its growth in several series of Plymouth Rocks, and not to be altered by sojourn in a single series of brown Leghorns. But as already mentioned, results entirely similar were obtained with the first Plymouth Rock fowls inoculated.

There are certain obvious physical differences between brown Leghorn and Plymouth Rock chickens to which the results might, conceivably, be due. The one breed is small and wiry, whereas the other is large and fat. Other things being equal, one might suppose that as hosts for all sorts of proliferating tissue the Plymouth Rocks would be better. To throw light on this point fowls of both varieties were inoculated with Chicken Tumor I, a simple spindle-celled sarcoma. This growth has already been mentioned for the striking specificity whereby its successful transplantation was at first confined to blood related Plymouth Rocks. It is now very malignant and grows well in chickens of many breeds.

Experiment 3.—Inoculations of Chicken Tumor I from a Plymouth Rock hen were made, as in experiments 1 and 2, into each breast of five Plymouth Rock and five brown Leghorn fowls. The results are given in text-figure 3.

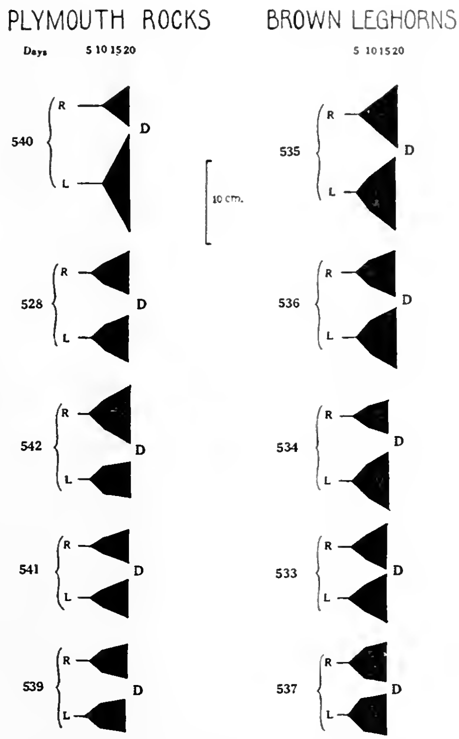
It will be seen (text-figure 3) that the tumor succeeded equally well in both varieties of fowls,—and this despite its early predilection for Plymouth Rocks. The findings with the rifted sarcoma can hardly be attributed then to gross physical differences in the chickens used.

Like our other transplantable chicken tumors the rifted sarcoma has a filterable agent as its cause. Yet in its transfer to new hosts, as carried out by introducing a bit of the fresh tumor tissue, a genuine transplantation is ordinarily involved, the new tumor arising by the survival and proliferation of the implanted fragment. The marked success of the sarcoma in hosts of an alien variety might conceivably be due either to an influence exerted on the transplanted cells or directly on the agent causing the disease. The results of a filtration experiment furnish some evidence for the latter view.[2]

[2] Rous, P., and Murphy, Jas. B., *Jour. Exper. Med.*, 1914, xix, 52.

Experiment 4.—Four Plymouth Rock fowls and four brown Leghorns were inoculated with equal portions of a Berkefeld filtrate prepared from the fresh tissue of the rifted sarcoma. A little sterile diatomaceous earth was added to the filtrate prior to its injection. The tumor used came from a Plymouth Rock fowl to which it had been transplanted after growing in two series of brown Leghorns.

The inoculated fowls were kept several months under observation. Two of the four Plymouth Rocks developed growths from which they eventually died. Of the four brown Leghorns only one developed a growth and this retrogressed.



TEXT-FIG. 3. Results of the inoculation of bits of the simple spindle-celled sarcoma into Plymouth Rock and brown Leghorn fowls. The tumor material was obtained from a Plymouth Rock.

No further experiments of the sort have been made because of the difficulty of obtaining the causative agent of the rifted sarcoma in active form. Unlike the agents causing our other chicken tumors it does not survive in the dried or glycerinated tumor tissue. Moreover, its activity in filtrates is very inconstant, and at best tumors are not produced until several months after the injection.

Among the numerous instances of the influence of race on the transfer of mammalian tumors we have been able to find but one in any way parallel to that here recorded. Tyzzer[3] transplanted a tumor of the Japanese mouse to hybrids of this breed and the ordinary white mouse, a variety completely insusceptible to the growth. In the F_1 generation of hybrids the tumor succeeded much better than in the Japanese mice. It may perhaps be remarked in this connection that Plymouth Rock fowls represent a mixture of several strains.

SUMMARY.

A transplantable sarcoma of the fowl, known as Chicken Tumor XVIII, in our series, succeeds better in chickens of an alien breed (Plymouth Rock) than in those of the variety in which it originated (brown Leghorn). This is not due to gross physical differences in the two breeds but to some more subtle factor and one which perhaps acts by influencing the agent causing the tumor. It would seem that Chicken Tumor XVIII, as it occurred in nature, was an instance of a disease appearing spontaneously in an animal of relatively insusceptible variety.

[3] Tyzzer, E. E., *Jour. Med. Research,* 1909, xxi, 519.

ON IMMUNITY TO TRANSPLANTABLE CHICKEN TUMORS.*

By PEYTON ROUS, M.D., and JAMES B. MURPHY, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

The observation that the transplanted tumors of mice sometimes retrogress has been provocative of much research; for in it the phenomenon of acquired resistance to neoplasms was first clearly recognized. Now we know that this resistance is not peculiar to tumors but is elicited by non-neoplastic tissues as well. A resistance attributable to a causative element in mammalian new growths has still to be demonstrated, as, indeed, has such an element. Causative agents for transplantable chicken tumors, on the other hand, have been found. Those thus far studied are filterable. In the light of this fact a comparison of the phenomena of resistance to chicken tumors and resistance to mammalian growths becomes of much interest; since it may well be that there exist gross differences that would prove the two of different etiology. The present paper is concerned with such a comparison. In addition there will be taken up the question of the relationship between the agents causing different chicken tumors as indicated by the specificity of the resistance to them.

We have used for the work three distinct chicken tumors, namely, a simple, spindle-celled sarcoma (Chicken Tumor I), an osteochondrosarcoma (Chicken Tumor VII), and a spindle-celled sarcoma curiously fissured with blood sinuses and showing a tendency to metastasize to the skeletal muscles (Chicken Tumor XVIII). Most of the data have been obtained with Chicken Tumor I, which has been longest in our hands.

NATURAL RESISTANCE.

Natural resistance to the avian tumors will be briefly dealt with, since it has already been reported upon in describing the growths.

* Received for publication, August 1, 1914.

Rat and mouse tumors, like the non-neoplastic tissues, can be successfully transferred under ordinary circumstances only to animals of the same species. This is true of chicken tumors as well. They will not grow in rats, mice, rabbits, or pigeons; and the spindle-celled sarcoma, the only one thus tested, will not grow in ducks. In fowls that are sick or emaciated the tumors do badly, either failing to develop after the inoculation, growing slowly, or retrogressing early. The same peculiarity has excited much attention in the case of mammalian growths. These latter grow best in young animals, and especially well in the new-born.[1] The influence of the age of the host upon chicken tumors has been tested only with the simple spindle-celled sarcoma. Young fowls have been found most susceptible as hosts for it, and in chick embryos it grows with extraordinary rapidity.[2]

Not a few mouse tumors are transplantable solely to animals of the variety in which the growth was spontaneous. A still greater specificity has been shown by Chicken Tumor I, which was transplantable at first only to blood relations of the original host and not to other varieties than the original until after months of propagation. The osteochondrosarcoma exhibits no preference for a special variety of fowl. The sarcoma rifted with blood sinuses shows what may be termed a reversed specificity, growing better in fowls of an alien sort (barred Plymouth Rock) than in the original brown Leghorn variety. This finding has been made the subject of a special paper.[3]

There exists an individual resistance to mammalian growths independent of all the factors thus far mentioned. Animals possessing it in its complete form fail to develop a tumor even though inoculated again and again. This is true of chicken tumors as well. But it is noteworthy in both cases that as the malignancy of the growth increases, owing to its sojourn in susceptible hosts, the number of animals insusceptible to it lessens. An individual naturally resistant to one form of mammalian tumor is frequently very susceptible to another. There is abundant evidence that this is true of avian growths as well.

[1] Unpublished work from this laboratory.

[2] Rous, P., and Murphy, Jas. B., *Jour. Am. Med. Assn.*, 1911, lvi, 741.

[3] Rous, P., and Lange, L. B., *Jour. Exper. Med.*, 1914, xx, 413.

Experiment 1.—Nine fowls were employed, four of them normal Plymouth Rocks, and the remainder brown Leghorns naturally resistant to the rifted sarcoma, as is shown by its failure to develop in them on a previous inoculation. All were now inoculated in the muscle of one leg with a bit of a slowly growing simple sarcoma (Chicken Tumor I), in the other with the rifted sarcoma. The inoculation of the simple sarcoma was unsuccessful and after seventeen days a second inoculation was made at the same site with more malignant material. The rifted sarcoma was then just beginning to appear. The final results are shown in text-figure 1.

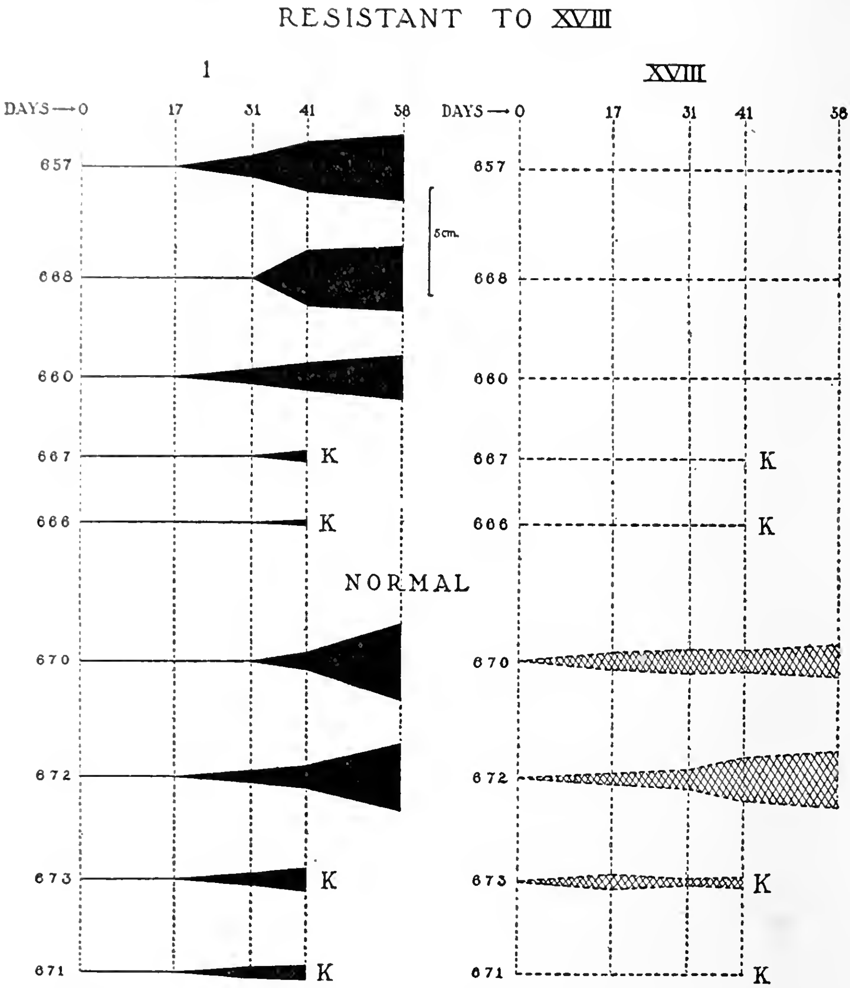
Text-figure 1 shows that fowls with a complete natural resistance to the rifted sarcoma and perhaps a slight acquired one (from the previous inoculation) were as susceptible to the simple sarcoma as normal fowls in which the rifted sarcoma grew well.

From all of the foregoing it is plain that the phenomena of natural resistance to chicken tumors are, in general, strikingly similar to those associated with rat and mouse tumors. The only apparent exception is in the tendency shown by the rifted sarcoma to grow better in fowls of an alien variety. Even here an instance somewhat similar may be found in mammals. Tyzzer bred together mice of two varieties, the one susceptible, the other insusceptible to a transplantable tumor of the Japanese waltzing mouse and found that the offspring of the F_1 generation were more susceptible than the susceptible parent. In the case of the rifted sarcoma the varieties of host tested were both the result of interbreeding several strains of fowls.

ACQUIRED RESISTANCE.

Some kinds of transplantable mammalian tumors grow progressively until the death of the animal; others after brief growth tend to become stationary and retrogress. The osteochondrosarcoma's behavior is of this latter sort. After a period of rapid enlargement as a chondrosarcoma in which spicules of bone gradually appear, it in most cases ceases to grow and is slowly absorbed. It not infrequently retrogresses after reaching a diameter of six or seven centimeters, but may take months to disappear, especially when it contains much bone. Only by the careful selection of tumors still growing has it been propagated. The simple spindle-celled sarcoma as a rule develops rapidly and progressively; but by the transplantation of slowly growing examples a retrogressing form may be obtained.

The rifted sarcoma develops slowly and with a considerable proportion of retrogressions.



Text-Fig. 1. Experiment 1. This shows that the simple sarcoma (Chicken Tumor I) implanted in fowls resistant to the rifted sarcoma (Chicken Tumor XVIII) grew as well as in normal fowls susceptible to the latter.

The time of appearance and rate of development of each tumor are shown by diagrams of which the width represents the diameter of the growth and the length its period of existence. A straight line indicates that no tumor developed. Cross-hatching indicates a rifted sarcoma, and solid black a simple sarcoma. The two are grouped in separate columns. The fowls are Nos. 657, 668, etc. K = killed.

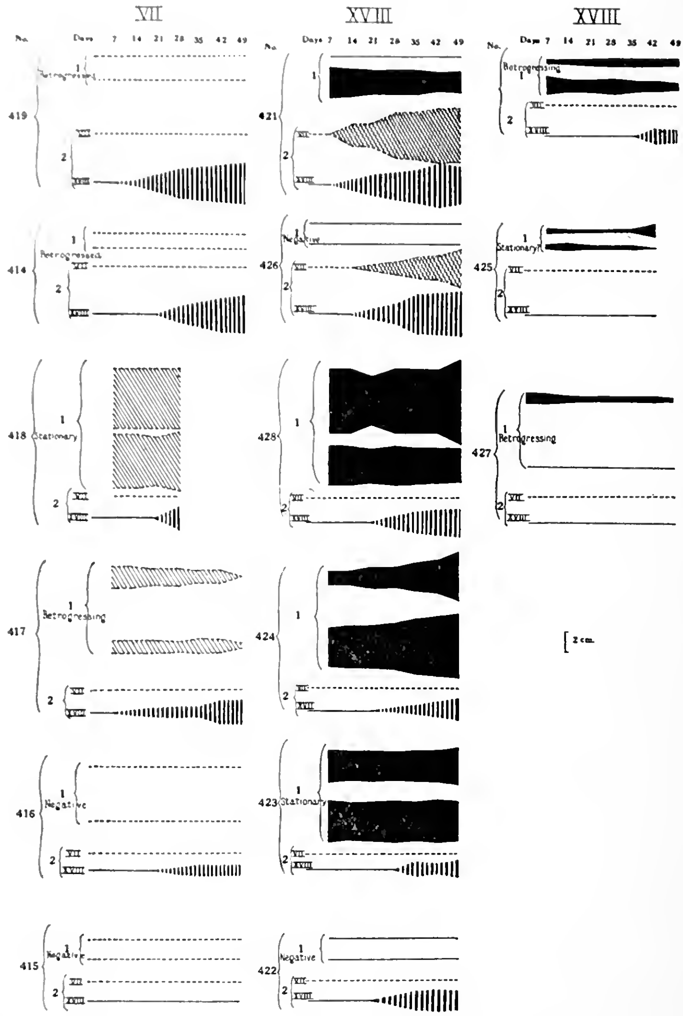
The amount of tumor material implanted has a marked influence on the course of all three chicken tumors. Retrogressing growths follow much more frequently the inoculation of single, small tumor bits than they do the inoculation of one to two cubic centimeters of the same tumor tissue, ground to a pulp. That dosage has an influence on the development and course of mammalian growths has long been known (Loeb, Clowes, and Baeslack).

Rats and mice in which tumors retrogress acquire resistance, as is shown by the fact that reinoculations within a few weeks usually fail of success. Some mouse tumors confer resistance on the host while they are still growing. This is especially true of tumors of retrogressing tendency. In the case of certain other neoplasms the factors which determine the course of the disease are so balanced that by mechanical means a stationary tumor may be made a growing one;[4] or reinoculations into a host in which the growth is stationary or retrogressing may be successful.

Exactly the same phenomena have been noted of the chicken tumors. The spindle-celled sarcoma grows rapidly and the success of secondary inoculations shows that it produces no notable concomitant resistance. The few individuals in which it is absorbed are usually resistant for a considerable time. The osteochondrosarcoma, a growth which tends to retrogress, produces a strong concomitant resistance (text-figures 2 and 5). Chickens in which it has been present for several weeks are always absolutely resistant on secondary implantation, and this at a period when the primary tumor is still growing. The slowly developing, rifted sarcoma often becomes stationary for long periods and then starts to grow again. By the use of malignant material hosts in which this growth is stationary or even slowly disappearing may sometimes be successfully reinoculated.

The resistance induced by the retrogression of a rat or mouse tumor is in part a pan-resistance but is most effectual against tumors of the same sort. Whether a pan-resistance to chicken tumors follows their retrogression has not been determined, but certainly much of the resistance is specific, as the following experiment shows.

[4] Loeb, L., *Jour. Med. Research,* 1901, vi, 28.



TEXT-FIG. 2. Experiment 2. This text-figure illustrates the fact that acquired resistance to the rifted sarcoma (Chicken Tumor XVIII) is slight as compared with that to the osteochondrosarcoma (Chicken Tumor VII); and it shows furthermore that resistance to the latter growth is to a large extent specific. All of the fowls (Nos. 419, 414, etc.) had been inoculated previously at two points. The character of this first inoculation (Chicken Tumor VII or Chicken Tumor XVIII) has determined the grouping in columns. For each fowl there are four diagrams representing tumor growth, or, in its absence, four lines. The diagrams bracketed together as 1 record the tumors of first inoculation; those bracketed as 2 record those of the second, the latter comprising an implantation with both Chicken Tumors VII and XVIII. The diagrams of the rifted sarcomata (Chicken Tumor XVIII) of first inoculation are given in solid black, those of the second in heavy hatching. A lightly hatched diagram indicates an osteochondrosarcoma of the first inoculation, and a cross-hatched one a tumor of the same sort following the second inoculation.

Experiment 2.—Six fowls previously inoculated with the osteochondrosarcoma and nine inoculated with the rifted sarcoma were chosen for this experiment. Some carried growths that were enlarging, some retrogressing growths, and others had shown themselves naturally resistant. All were now inoculated with the rifted sarcoma in the wing muscles of one side and with the osteochondrosarcoma at the same spot on the other side. 0.1 c.c. of a suspension of the fresh tumor tissue in Ringer's solution was used in each case. The course of the old tumors and the development of the new are shown in text-figure 2.

It will be seen from text-figure 2 that all of the fowls previously inoculated with the osteochondrosarcoma were now resistant to it. The malignancy of the material employed is proved by the rapidity with which it gave rise to tumors in two fowls previously implanted with the rifted sarcoma. This latter tumor grew in all but one of the fowls resistant to the osteochondrosarcoma. It also grew in seven of the nine hosts previously inoculated with a growth of its own sort. In one fowl the tumor of the first inoculation was actually retrogressing while that of the second enlarged.

When implanted simultaneously in the same host the chicken tumors preserve their character unchanged. The simple sarcoma metastasizes, as usual, to the lungs and other viscera, and the rifted sarcoma still gives secondary growths in the muscles, the source of each dissemination being clearly traceable from its histology. Sometimes one tumor grows rapidly whereas the others do badly or fail to grow (text-figure 4). So too it is with neoplasms of the rat and mouse. In a previous article the fact has been pointed out that the histological signs of resistance to these latter are identical with those to chicken tumors when allowance is made for the peculiarities of the two classes of host.[5]

Despite the efforts of many workers an immune principle effective against rat and mouse tumors has yet to be demonstrated in the blood of animals recovered from these growths. Crile and Beebe[6] succeeded in curing dogs of infectious lymphosarcoma by transfusing to them blood from other dogs in which the growth had retrogressed; but the lymphosarcoma has characters which distinguish it from the true neoplasms. Nevertheless, attempts to cure chicken tumors by means of transfusion have seemed advisable. Five fowls

[5] Rous, P., and Murphy, Jas. B., *Jour. Exper. Med.*, 1912, xv, 270.

[6] Crile, G. W., and Beebe, S. P., *Jour. Med. Research*, 1908, xviii, 385.

in which a relatively non-malignant form of the simple sarcoma was developing as the result of inoculation were bled from thirty-five to sixty-five cubic centimeters and an equal or slightly larger amount of blood was transfused to them from resistant fowls. In these latter the simple sarcoma had retrogressed and several intraperitoneal inoculations of sarcomatous tissue had from time to time been made without yielding tumors, a fact confirmed at autopsy. Transfusion was done at a time when resistance to the sarcoma may be supposed to have been at its greatest, that is to say, some two to three weeks after a massive injection of sarcomatous tissue. But in the fowls receiving the blood the tumors grew quite as well as in untransfused controls.

It is well known that not only does the retrogression of a mammalian tumor render the host unfavorable for subsequent tumor grafts but that injections of normal tissues, of normal blood even, will act to this end. Embryonal tissue is especially effective. In our experience the injection of hashed chick embryo does not confer resistance to the spindle-celled sarcoma of the fowl. But the tumor used was very malignant and may not have been sufficiently sensitive as an indicator.

Thus far the chicken tumors have been considered simply as transplantable new growths. The phenomena of acquired resistance to them resemble such as are seen under like conditions in the case of mammalian growths and suggest no more than these the presence of a causative agent distinct from the tumor cells.

RESISTANCE TO THE TUMOR-PRODUCING AGENTS.

By a special method there have been demonstrated two distinct forms of resistance against the simple sarcoma when it is transferred by grafting,—the one directed against the transplanted tumor cells, the other against the growth's causative agent.[7] Resistance of the latter sort will come into consideration in the findings now to be discussed.

With the exception of Königsfeld[8] workers with mammalian tumors have found that neoplastic tissue killed by drying fails to in-

[7] Rous, P., *Jour. Exper. Med.*, 1913, xviii, 416.

[8] Königsfeld, H., *Centralbl. f. Bakteriol.*, 1te Abt., *Orig.*, 1914, lxxiii, 316.

duce resistance against subsequent grafts. We have repeatedly attempted to induce with dried tissue resistance to the spindle-celled sarcoma of the fowl. The growth's causative agent remains active after drying, so it was necessary to make the first inoculations with material rid in some way of its tumor-producing property. The dried and powdered tissue was taken up in distilled water, heated at 60° C. for fifteen minutes, and injected intraperitoneally. For the later injections material submitted to 55°, 53°, or 50° C. for fifteen minutes, and finally unheated material, was used. Several groups of fowls were employed, but few came to the eventual test with the implanted growth, because nearly all developed tumors following the inoculation with unheated, dried tissue. Those remaining may well have been naturally resistant. If any protection is elicited by the injection of dried material in which the agent exists in attenuated form, it must certainly be very slight.

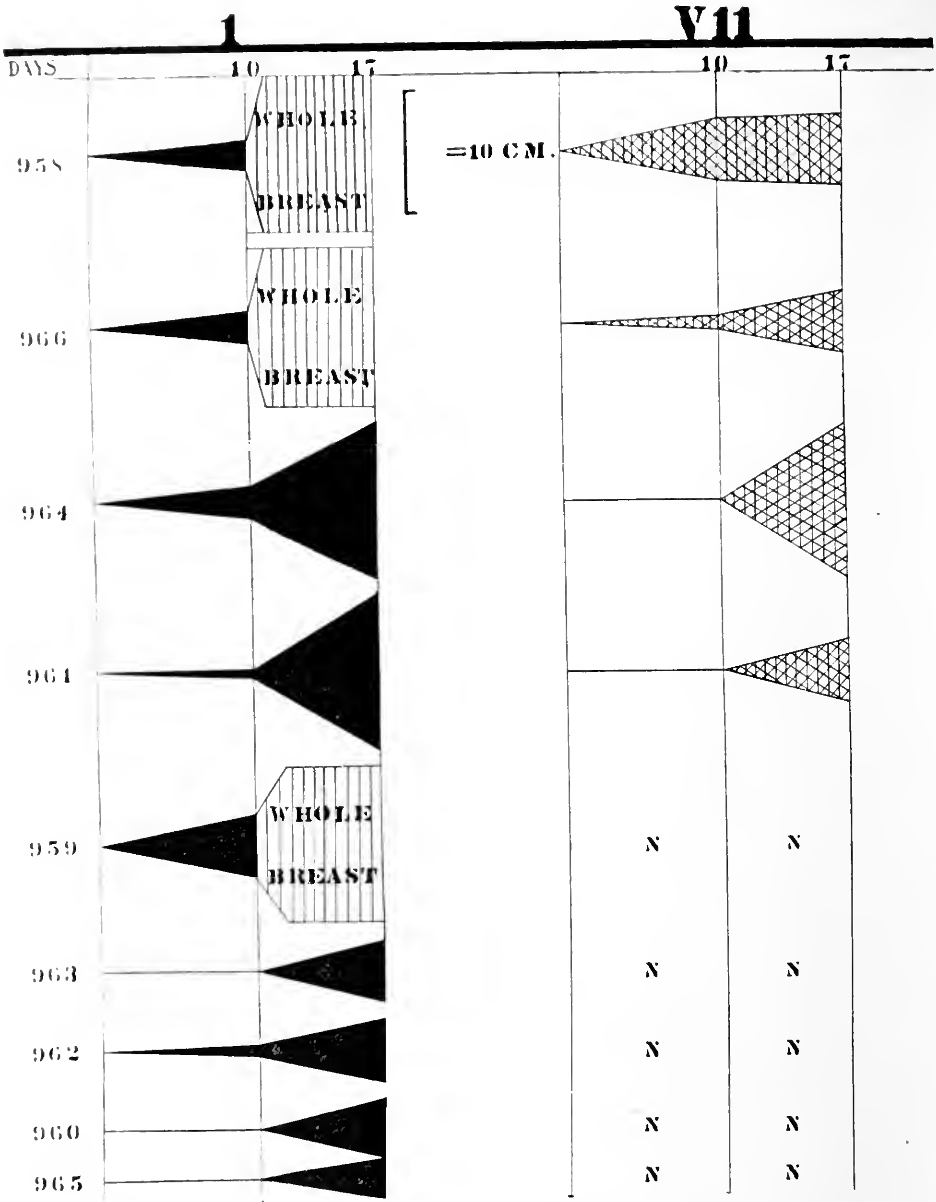
SPECIFICITY OF THE RESISTANCE.

That there exists a natural resistance to the agents is shown by their failure to produce tumors in some hosts. The question arises as to how far this resistance is specific.

Experiment 3.—Nine healthy Plymouth Rock fowls were inoculated, in one breast with 0.5 c.c. of a suspension of the dried tissue of an osteochondrosarcoma (Chicken Tumor VII), in the other breast with 0.1 c.c. of a like suspension of the dried spindle-celled sarcoma (Chicken Tumor I). The suspensions were made by rubbing up 1 gm. of dried tumor tissue in 9 c.c. of distilled water. The difference in dosage was to compensate for differences in the malignancy of the tumors. The results will be found in text-figure 3.

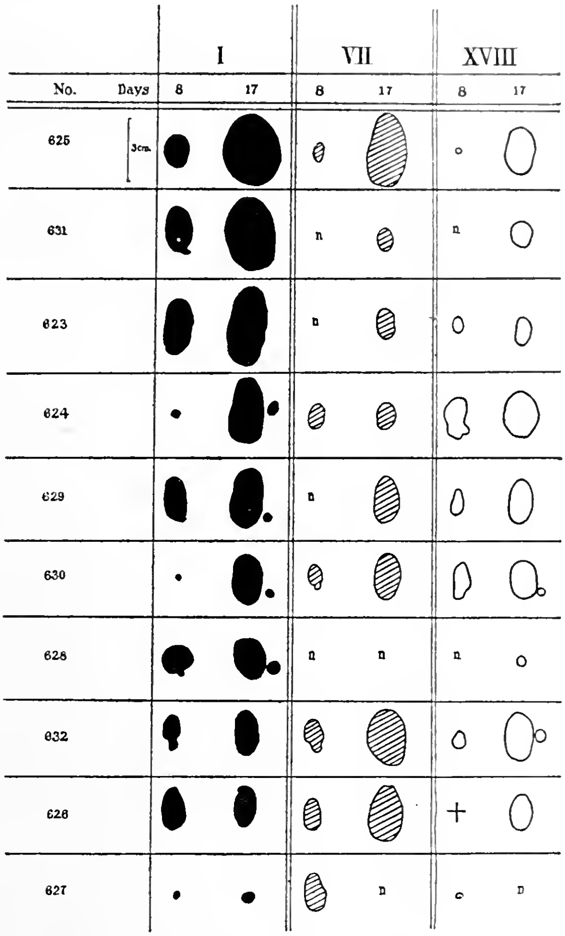
It is evident from text-figure 3 that the agents of the simple sarcoma and the osteochondrosarcoma are largely influenced by the same factors of natural resistance. In the experiment which it illustrates the period which elapsed before the appearance of a palpable tumor was so nearly the same for the two growths that the results can scarcely be referred to concomitant resistance induced by one tumor and effectual on the other.

With the rifted sarcoma a test of the above sort has not been possible because its agent is obtained apart from living cells only inconstantly and with difficulty. Comparative transplantation has been resorted to but this introduces a factor of error in that there



TEXT-FIG. 3. This shows that the same factors of natural resistance influence the activity of the agents causing two different chicken tumors. The fowls (Nos. 958, etc.) were inoculated in one breast with dried material of the simple sarcoma (Chicken Tumor I), in the other with that of the osteochondrosarcoma (Chicken Tumor VII). The diagrams are black for the simple sarcoma, cross-hatched for the osteochondrosarcoma.

are transferred with the agent tumor cells strange to the new hosts yet capable of active proliferation in many of them. With such a large disturbing element one would scarcely expect to learn much regarding the specificity of resistance to the agents. The following experiment gives evidence for the correctness of this view.

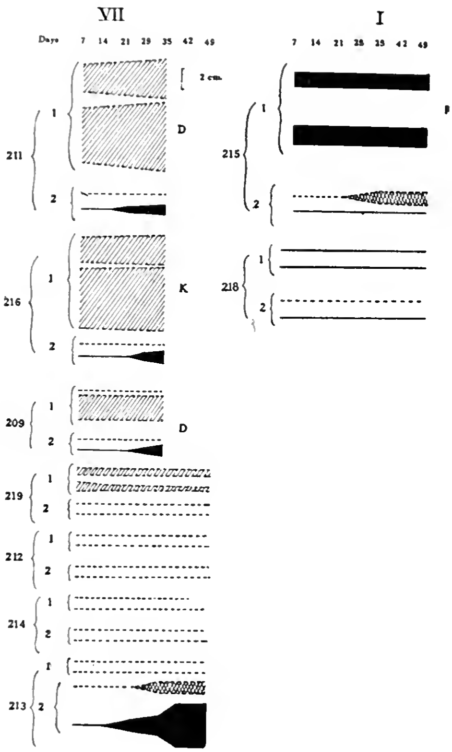


TEXT-FIG. 4. Fowls Nos. 625, etc., were inoculated simultaneously at different points with all three chicken tumors (I, VII, and XVIII). The text-figure shows that the growths varied independently of one another.

Experiment 4.—Ten healthy Plymouth Rock fowls were inoculated at different spots with all three chicken tumors (I, VII, and XVIII) in the amount of

0.1 c.c. of the finely ground fresh tissue. The sites chosen were in the muscle of both breasts, and the arrangement of the inoculations was varied from fowl to fowl. The results are shown in text-figure 4.

It will be seen from text-figure 4 that the tumors varied independently of one another. The findings as regards the simple sarcoma and the osteochondrosarcoma give no hint of the relationship seen in text-figure 3.



TEXT-FIG. 5. This text-figure has to do, like text-figure 2, with reinoculations; and the same general explanation holds good for it. The results of the first inoculation are given in the bracket 1 and those of the second in 2. The black and hatched diagrams are those of the simple sarcoma and the osteochondrosarcoma, respectively. The second inoculation was made with dried material of each growth. It will be seen that the agent of the simple sarcoma failed to give rise to tumors in fowls in which this growth had done badly on previous inoculation, whereas it caused growths in fowls resistant to the osteochondrosarcoma. The resistance against the latter tumor growth is also largely specific. Of seven fowls previously inoculated with it but one was susceptible on second inoculation. This fowl, No. 213, was supposed to be naturally resistant because of an unsuccessful inoculation some weeks previously, but the agents of both tumors engendered growths in it.

SPECIFICITY OF ACQUIRED RESISTANCE TO THE AGENTS.

Obviously the resistance acquired by a fowl in which a tumor has retrogressed must be effectual not only against the tumor cells but against the associated agent,—else this latter by acting on the cells of the host would produce a tumor. The following experiment indicates that acquired resistance to a tumor-producing agent is largely specific.

Experiment 5.—One fowl in which the simple sarcoma had retrogressed, one in which it was stationary, and four fowls carrying the osteochondrosarcoma were employed. They were inoculated, in one breast with 0.1 c.c. of a thin paste made by rubbing up dried tissue of the simple sarcoma with Ringer's solution, in the other with 0.5 c.c. of a similar paste of the dried osteochondrosarcoma.

As text-figure 5 shows, the four fowls carrying the osteochondrosarcoma evinced a complete resistance to it on secondary inoculation, whereas the simple sarcoma developed in three of them. The opposite result was obtained with the fowls in which the simple sarcoma had retrogressed or was stationary. Both now proved resistant to this growth, but in one the osteochondrosarcoma developed. The fact that the agent of the osteochondrosarcoma is relatively inactive renders the result more striking.

SUMMARY.

The phenomena of natural and acquired resistance to transplanted chicken tumors strikingly resemble those observed in the case of transplanted mammalian growths; and no more than those do they suggest that the tumors have an extrinsic cause.

That there may exist in fowls implanted with a chicken tumor a resistance directed against the tumor-causing agent distinct from the resistance manifested against the alien tumor cells has been shown in a previous article.[9] Both sorts of resistance are present in a fowl in which a tumor has retrogressed, the resistance in such an instance being acquired. That directed against the agent is largely specific, giving little if any protection against the agents

[9] Rous, P., *loc. cit.*

causing other tumors. There is some evidence that the conditions upon which a fowl's natural resistance depends are the same for the agents causing different chicken tumors.

It has proved impossible to protect chickens against the agent causing the simple sarcoma by injecting them with dried tumor material in which this agent has been attenuated by heat. The transfer of blood from resistant fowls to fowls with growing tumors is in our experience void of effect on the tumors.

THE INFLUENCE OF DIET ON TRANSPLANTED AND SPONTANEOUS MOUSE TUMORS.*

By PEYTON ROUS, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

PLATE 21.

That cancer progresses relatively slowly in emaciated, old people has long been known; but the influence on the disease of general nutritive conditions as thus indicated has only lately attracted wide attention, following the observation that some strains of transplantable tumors grow badly, or not at all, in emaciated hosts. Moreschi[1] was the first to study the phenomenon systematically. He found that grafts of mouse sarcoma grew less frequently and more slowly in animals losing weight on a low diet. But, though he thus demonstrated that malnutrition of the host affects adversely bits of strange tissue as yet unvascularized and unattached, he did not take up the influence of the factor on large growths, on recurrences, and on the development of metastases, or, in other words, on neoplastic conditions such as require palliative treatment in human beings. Experiments in this direction which I began[2] shortly after Moreschi's paper was published demonstrated the fact that a tumor which does badly when transplanted to hosts previously underfed may be quite uninfluenced by dieting begun after the growth is vascularized and has started to develop. Nodules of the Flexner-Jobling rat carcinoma, a few millimeters in diameter, grew with equal rapidity in hosts emaciating on a restricted ration and in controls gaining weight on the same sort of food (text-figures 1 and 2). The Jensen rat sarcoma, on the contrary, even after it had reached a large size, was retarded in its development by under-

* Received for publication, August 1, 1914.

[1] Moreschi, C., *Ztschr. f. Immunitätsforsch., Orig.*, 1909, ii, 651.

[2] Rous, P., *Proc. Soc. Exper. Biol. and Med.*, 1910–11, viii, 128.

feeding the host. But the forces which determine the course of this tumor must be delicately balanced, for it often retrogresses without evident cause in whole series of healthy rats.

Not only have general nutritive conditions an influence on some transplanted tumors, but the character of the host's food may be important. Van Alstyne and Beebe[3] have shown that tumor grafts

CONTROLS						FOOD LIMITED					
NO.	WEIGHT		TUMOR			NO.	WEIGHT		TUMOR		
	INITIAL	CHANGE	INITIAL	AFTER 1 WK.	AFTER 2 WKS.		INITIAL	CHANGE	INITIAL	AFTER 1 WK.	AFTER 2 WKS.
13	50	+10				1	60	0			
14	50	+25				2	70	0			
15	75	+25				3	75	+5			
16	70	+15				4	65	0			
17	75	+20				5	70	−5			
18	90	+30				6	100	−20			
19	80	+25				7	70	−15			
20	50	+20				8	85	−15			
21	75	+20				9	70	−5			
22	65	+25				10	100	−10			
23	75	+15				11	60	−10			D
24	90	+25				12	70	−10			D

TEXT-FIG. 1. The Flexner-Jobling adenocarcinoma, once it has begun to develop, grows with equal rapidity in rats gaining weight on a mixed diet and those underfed on the same food.

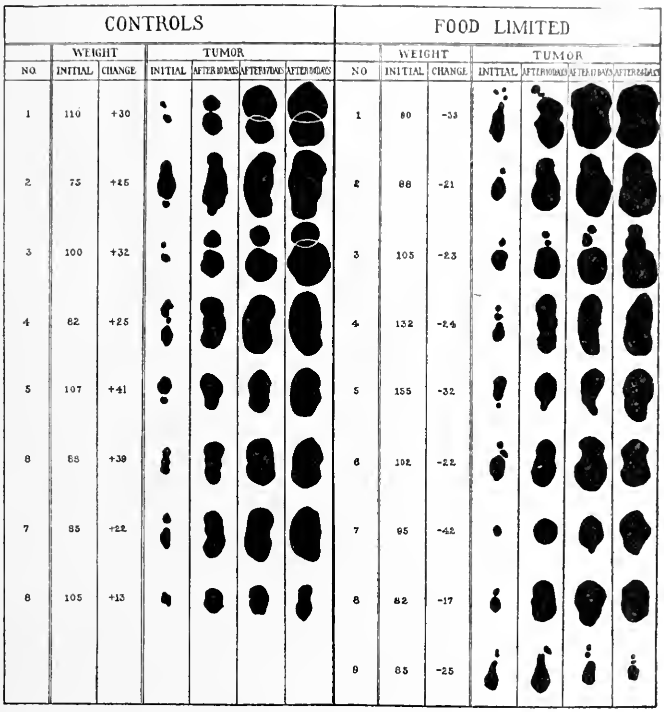
give rise relatively seldom to growths in animals fed for some time beforehand on a non-carbohydrate diet. Sweet, Corson-White, and Saxon[4] kept mice on a modification of a food devised by Mendel and Osborne[5] that prevents the growth of rats because of the lack of certain constituents; and they found that a mouse car-

[3] Van Alstyne, E., and Beebe, S. P., *Jour. Med. Research,* 1913–14, xxix, 217.

[4] Sweet, J. E., Corson-White, E. P., and Saxon, G. J., *Jour. Biol. Chem.,* 1913, xv, 181.

[5] Osborne, T. B., Mendel, L. B., and Ferry, E. L., *Ztschr. f. physiol. Chem.,* 1912, lxxx, 307.

cinoma had poor success in such hosts. Unfortunately it is not certain whether the results of these investigations are to be attributed to a specific lack in the foods employed or to the circumstances that the diet of the specially fed hosts differed from that of the animal furnishing the tumor transplanted to them. That such a difference may adversely affect tumor grafts has repeatedly been shown[6]; and



TEXT-FIG. 2. This records findings similar to those of text-figure 1. But the dieting was begun when the tumors were larger and was continued for a longer time. The underfed animals lost greatly in weight.

it was with tumor grafts that the investigators mentioned obtained their positive results. Van Alstyne and Beebe found a non-carbohydrate diet ineffective when it was begun on the day that the tumor was implanted. Sweet and his collaborators made experiments only with tumor grafts.

[6] Haaland, M., *Berl. klin. Wchnschr.*, 1907, xliv, 713. Stahr, H., *Centralbl. f. allg. Path. u. path. Anat.*, 1909, xx, 628.

Altogether the work mentioned shows that the development of tumor grafts can in many cases be prevented or retarded by underfeeding the host or by putting it on a special diet. By the first method certain transplantable tumors can be checked or retarded in their course even after they have reached a large size. The method is ineffectual with established growths of one kind at least, the Flexner-Jobling rat carcinoma, despite the fact that grafts of this tumor, as yet unvascularized, are easily influenced. Whether recurrences and metastases of such a growth will behave like the parent tumor, or, like new made grafts, prove sensitive to influences exerted through the diet is not yet known; nor has the influence of special diets on large tumors been adequately looked into. Apart from certain suggestive clinical instances nothing is known as to the influence on spontaneous growths of alterations in the food of the host. The present study is concerned with some of these points.

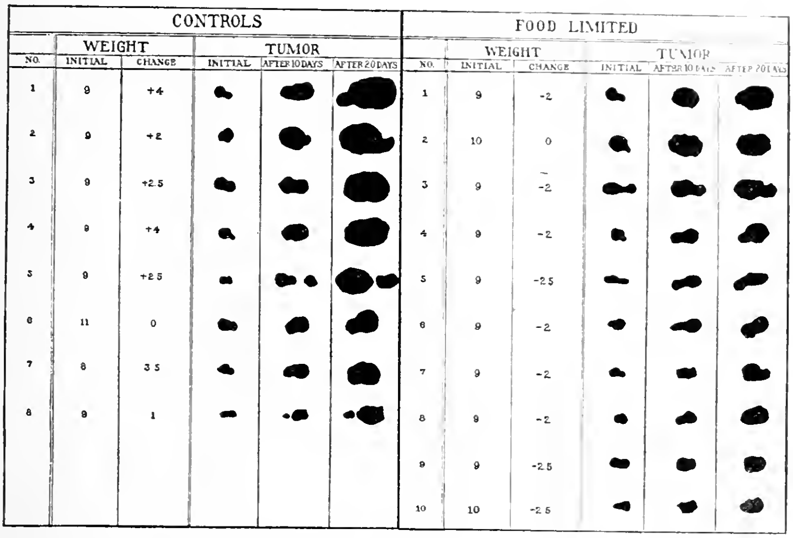
THE INFLUENCE ON TRANSPLANTED TUMORS.

Several mouse tumors and the Flexner-Jobling adenocarcinoma of the rat were used. The mice which served as hosts were not merely underfed so that they lost weight but were given as their sole diet Sweet's modification of Mendel's food.[7] It was thought that in this way more outspoken results might be obtained. Rats, as in previous experiments with the Flexner-Jobling growth, were underfed on a standard bread compounded of oatmeal, corn-meal, rye flour, milk, and sugar with a measured quantity of fresh milk to moisten it. Experiments later to be described show that Sweet's food has no special influence on this tumor.

In the attempt to learn whether the development of metastases can be influenced by diet, experiments were carried out with two metastasizing mouse carcinomata, kindly furnished by Dr. Tyzzer and by Dr. Bashford, respectively. One was a growth of the Japanese waltzing mouse, the other strain 63 from the laboratories of the Imperial Cancer Research Fund. But as both these tumors, even after they had reached a considerable size, were markedly checked in their growth by underfeeding the host on Sweet's

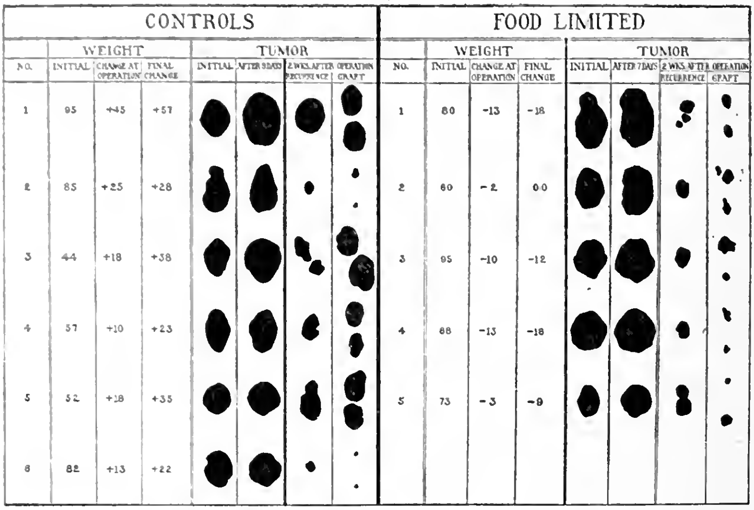
[7] A food composed of starch, lard, lactose, agar, a salt mixture, and gluten obtained by washing wheat flour.

food (text-figure 3), it seemed useless to observe the effect of the treatment on secondaries, since the number of these secondaries is of course directly dependent on the activity of the primary neoplasm. The Flexner-Jobling tumor could not be utilized because it had ceased to metastasize with sufficient regularity. A strain of mouse carcinoma (native strain No. 33) which never gives secondaries, was tested and found to be markedly held back by the dieting.

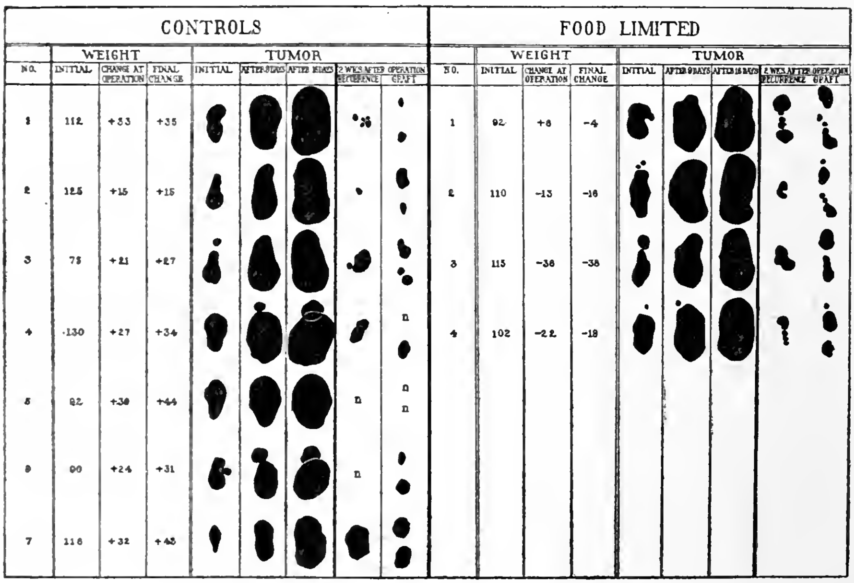


TEXT-FIG. 3. A transplantable adenocarcinoma of the Japanese waltzing mouse was markedly retarded in its growth by underfeeding the host on Sweet's food. The diet was begun after the tumors had reached a considerable size. The controls were full fed on a mixed ration.

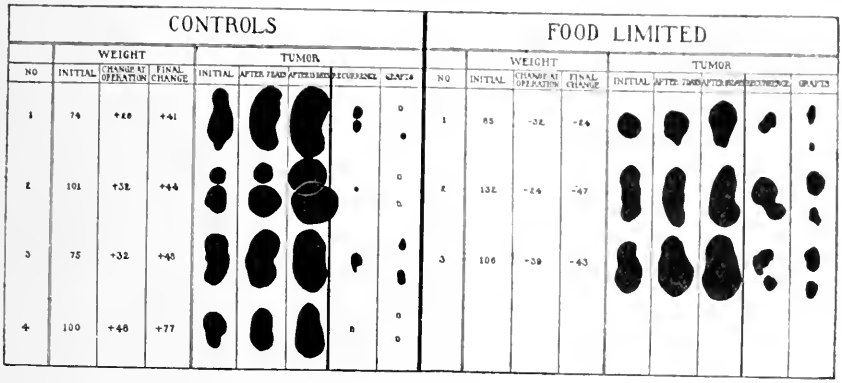
The effect of underfeeding upon recurrences was studied with the Flexner-Jobling adenocarcinoma. Just as three years previously, this growth was uninfluenced by changes in the nutritive condition of the host instituted after it had reached considerable size (text-figures 4, 5, and 6). Rats about three fourths grown with subcutaneous tumors resulting from inoculation of the same material were paired against one another according to body-weight and size of the tumors, and one of each pair was underfed so that it grad-



TEXT-FIG. 4.



TEXT-FIG. 5.



TEXT-FIG. 6.

TEXT-FIGS. 4, 5, AND 6. These give the results of three experiments with the Flexner-Jobling adenocarcinoma, designed to test the effect of underfeeding on recurrences and operative disseminations (grafts) of this growth. Half of the animals were underfed on a mixed diet, half full fed; and after several weeks all were operated upon. The tumor was removed save for a small fragment, and two auto-implantations were made in the subcutaneous tissue.

In one series of underfed animals (text-figure 4) the recurrences and grafts grew less well than in the controls; in a second series (text-figure 5) no notable differences were observed; while in the third growth was far better in the underfed animals.

ually lost in weight. The controls were full fed on the same diet and grew rapidly. After several weeks of dieting, when the experimental animals had become thin, all were operated upon by a uniform procedure. The tumor was removed save for a fragment about one and one half millimeters in diameter, which was left with its vascular connections intact; and two autotransplantations were made of small bits to the subcutaneous tissue of the flanks. The feeding was not changed. The results varied strikingly from experiment to experiment. In one series of thin animals the development of recurrences and grafts was much delayed, in another it occurred with the same rapidity as in the controls, and in a third it was much more rapid (text-figures 4, 5, and 6).

Evidently in these experiments with the Flexner-Jobling tumor factors sufficient to cause a complete reversal in the findings were uncontrolled. Without doubt one such factor is the resistance elicited in the host by the implanted alien tissue. This resistance

has been often studied. That it may lead to the retrogression of transplanted tumors, even when large, or prevent recurrences of them is well known. Through the instability that it introduces it may have been responsible for the sensitiveness to alterations in the host's diet shown by the transplanted mouse tumors used in the present work. Against spontaneous tumors such a resistance has not been demonstrated despite repeated attempts. So the findings with transplantable tumors can hardly be taken as a basis for generalizations regarding the effect of diet on spontaneous growths.

THE INFLUENCE ON SPONTANEOUS TUMORS.

Some hundred and fifty spontaneous mouse tumors were obtained for the work. All had arisen as lumps in the mammary glands of old females, and all except three were carcinomata, these three being examples of carcinoma sarcomatodes. The histology of the growths was varied.

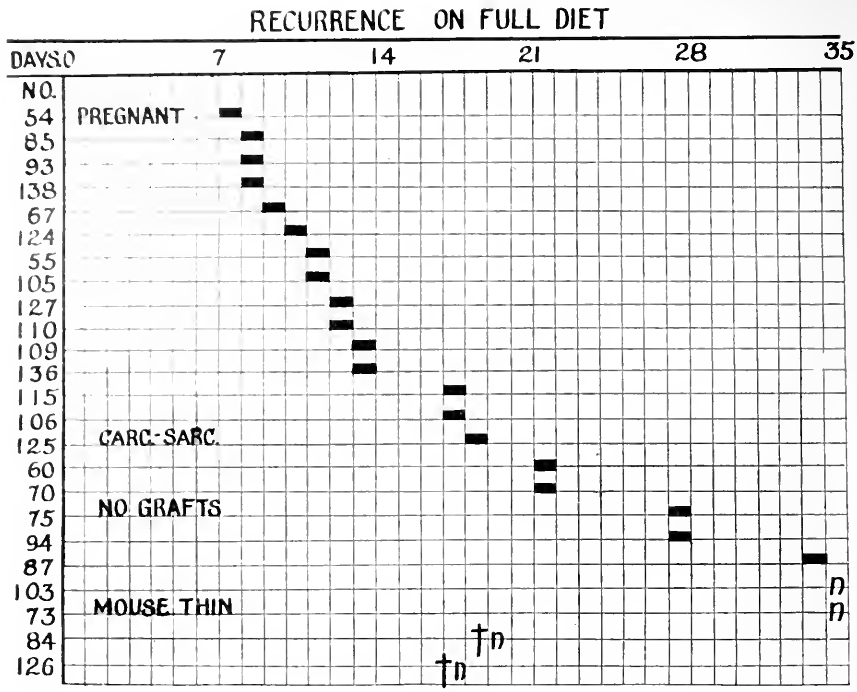
The tumor mice were divided into three groups of the same general character. One group which served as control was full fed on ordinary diet (bread and milk, grain); a second was underfed on Sweet's modification of Mendel's food for some days prior to operation and thereafter throughout the term of observation; and the third was given ordinary diet before operation, and an abundance of Sweet's food afterwards. Each animal had a jar to itself, provided with a water bottle. As in the experiments with the Flexner-Jobling carcinoma the tumors were removed save for a fragment about one and one half millimeters in diameter which was left with its vascular connections intact; and two autotransplantations of similar bits were made into the subcutaneous tissue of the animals' flanks. These latter bits may be taken to represent in some sort disseminations at the time of operation. The wounds were closed with very fine silk and usually healed by first intention. When there were multiple tumors, as frequently happened, all save one were taken out *in toto,* and a fragment of this one was left and implantations made as usual. The mice were kept for thirty-five days after operation and weighed and examined frequently. Haaland[8] has found

[8] Haaland, M., *Fourth Scientific Report of the Imperial Cancer Research Fund,* 1911, 1.

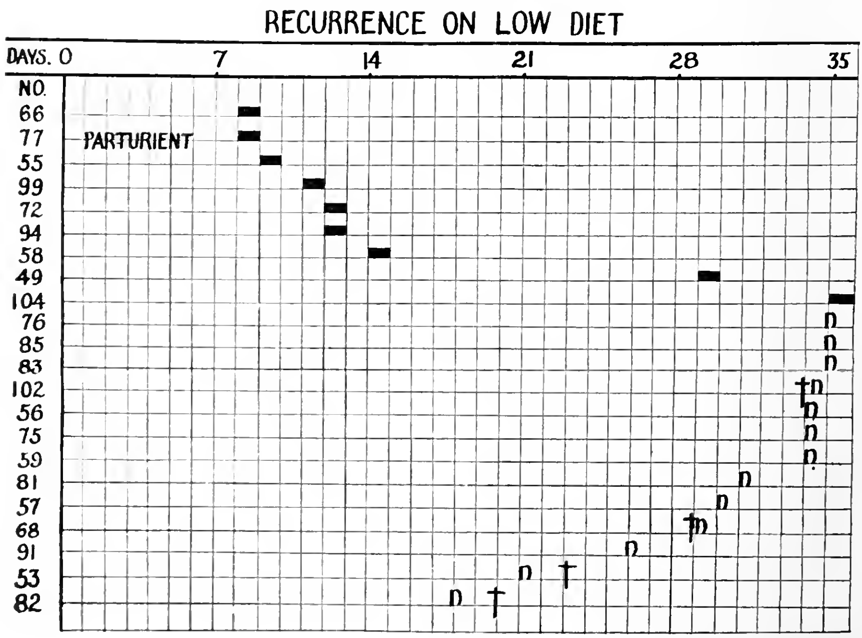
that grafts of spontaneous mouse tumor implanted in the animal furnishing the growth usually began to develop within thirty-five days. Text-figure 9 confirms his findings.

Many complications and deaths occurred among the operated mice, diminishing the number that could be used for a comparison. Some of the animals had very large tumors and were already emaciated. These were ruled out. In the charts are figured the results in mice surviving two weeks or longer without wound infection, or severe illness, or gaping of the wound that would affect the recurrence. As was to have been expected, much the largest proportion of deaths from intercurrent causes was in the underfed mice. Constant vigilance was needed to ensure that the food they received was sufficient to support life, and yet so little that they lost in weight. Minor ills such as otitis, scabby skin, and conjunctivitis were about equally frequent in the three series. Recurrences and growth of the grafts were first recorded as such when growing nodules larger than the original tumor fragments had made their appearance. The recurrences usually developed from the tumor bit left in the wound; but often, especially in the control group, they were multiple, or diffuse in the scar. Owing to the way in which the operation was carried out tumor cells must have been scattered throughout the wound in practically every case.

A comparison of text-figures 7 and 9 with text-figures 8 and 10 shows that in the mice losing weight, as a result of underfeeding with Sweet's modification of Mendel's food, recurrences and the growth of tumor grafts were delayed in most cases and prevented in many throughout the term of observation. In 83 per cent. of the twenty-four mice on ordinary diet a recurrence was noted, and in only 41 per cent. of the twenty-two underfed mice. The effect on growth of the grafts was somewhat less marked. They grew in 68 per cent. of the controls and 41 per cent. of the underfed animals, appearing on the average fifteen days after operation in the former and twenty-five days after it in the latter. In the controls the new tumors enlarged much more rapidly, and coalescing recurrences throughout the healed wound were relatively frequent. A few of the tumors in the underfed animals,—and these the most malignant histologically,—were little if at all affected by the dieting.

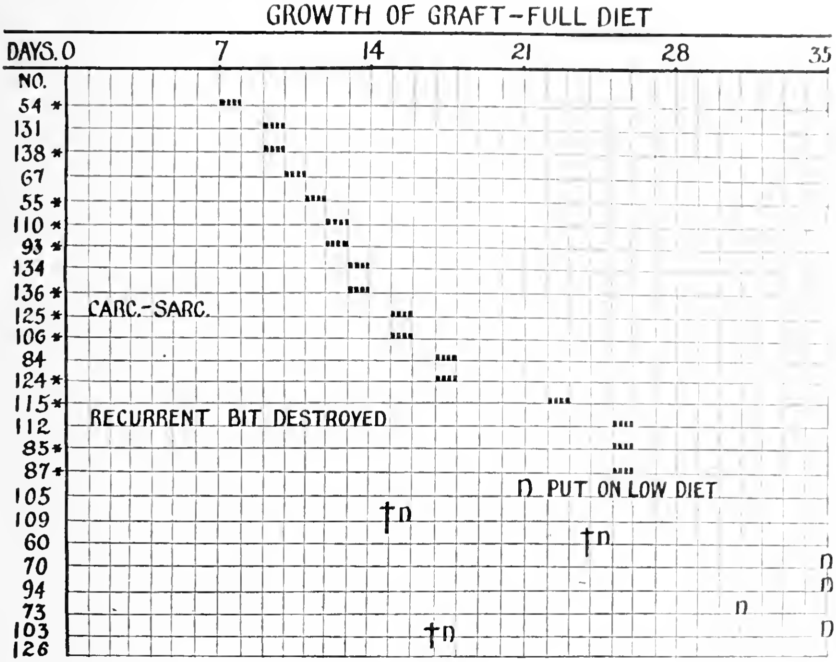


Text-Fig. 7.

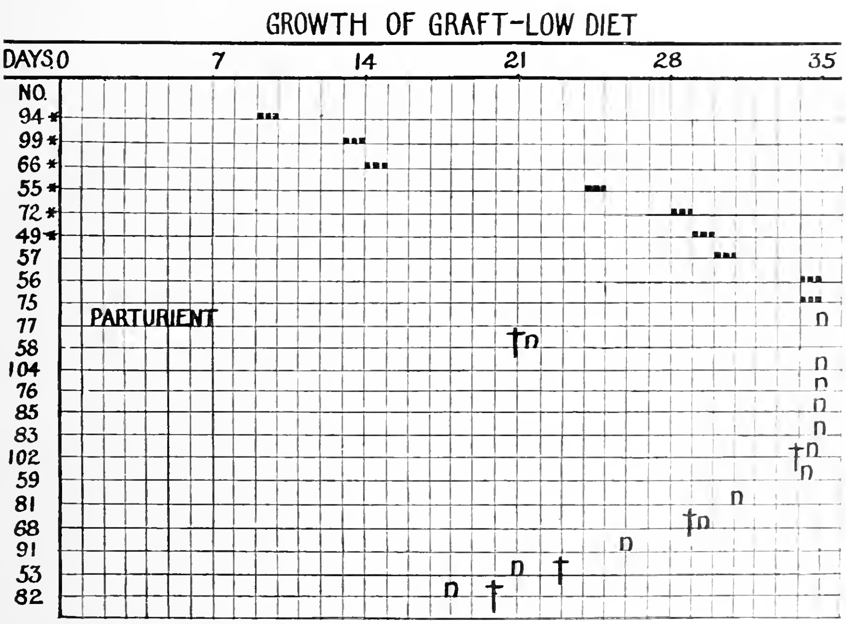


Text-Fig. 8.

Text-Figs. 7 and 8. These give the time of appearance of recurrences of the spontaneous tumors of a series of mice fed abundantly on a mixed diet, and of another series underfed before and after operation on Sweet's food. In the first column are the numbers designating the mice. The distance of the black dashes from the ordinate indicates how soon after operation the recurrences appeared. N = no recurrence.



TEXT-FIG. 9.



TEXT-FIG. 10.

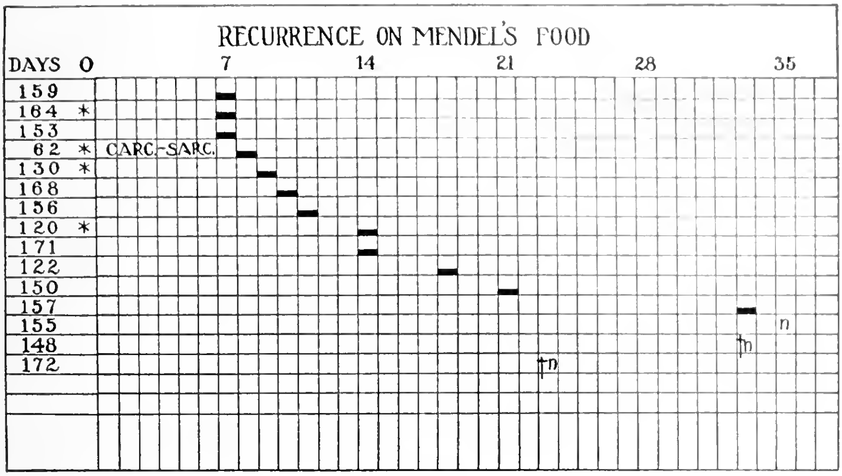
TEXT-FIGS. 9 AND 10. The time of appearance of growing grafts in the mice of text-figures 7 and 8. A star after the number of a mouse indicates that in this animal there was a recurrence as well.

as far as could be observed. Recurrences of these growths took place and grafts of them enlarged with practically the same rapidity as in the most malignant instances among the controls.

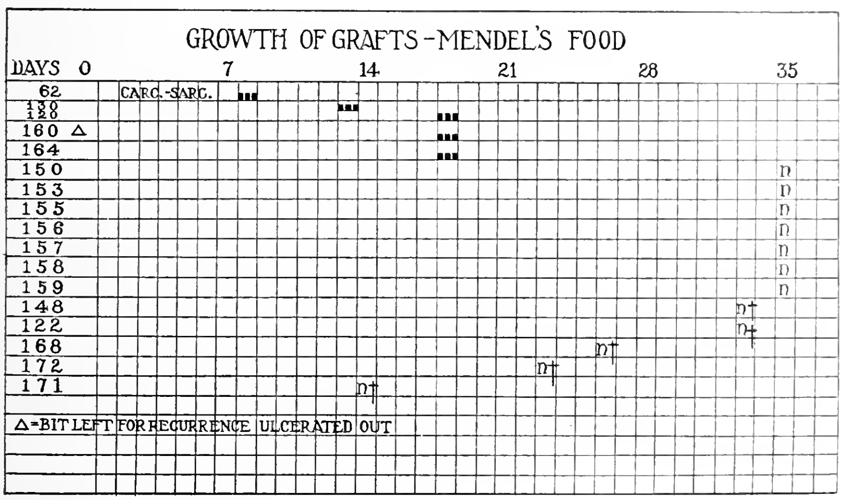
The treatment that secured these results in the dieted mice was drastic. By the time of operation the animals had lost, on the average, 12 per cent. of their weight and at the end of the thirty-five days 24 per cent. But as most were fat when obtained, the actual emaciation was less than is suggested by the figures. Toward the end of the fourth week the mice showed almost regularly an aversion for the special food, and it became necessary to supplement this with a little bread and milk if they were to remain alive.

The results were not permanent in that cures were effected. Some of the mice which showed no sign of tumor during the period of underfeeding were put on ordinary diet at its completion, and practically without exception they developed recurrences or growing grafts, or both, soon after regaining their weight. Some of the control animals in which the tumor had reappeared were transferred to the underfed group, and when they had begun to lose weight were operated upon a second time according to the usual procedure. Occasionally the results were striking. A carcinoma solidum which recurred on the control diet eight days after operation failed to reappear in the thirty-six days in which the host was underfed following a second operation, only to recur again, and within four days, when the control diet was resumed. In this animal the grafts did not grow.

It has been of special interest to determine whether results similar to those in the underfed group could be obtained in mice kept in nitrogenous equilibrium on the special diet. But in the work to this end an unexpected obstacle was met with. Rats stay in good health and retain their weight for long periods when fed on Mendel and Osborne's food; but on Sweet's modification of this as prepared in our laboratory mice were found to do badly. For example, nine healthy, adult mice weighing an average of twenty grams lost an average of 4.4 grams during three weeks in which they were given the food in abundance. Young animals fare even worse. Some old females maintain equilibrium for ten days or two weeks, but the majority begin to lose weight within a few days and by the



TEXT-FIG. 11.



TEXT-FIG. 12.

TEXT-FIGS. 11 AND 12. The time of appearance of recurrences and grafts, respectively, of the spontaneous tumors of mice fed abundantly on Sweet's food from the time of operation. In text-figure 11 a star after the number of a mouse indicates that the grafts also grew.

end of the fourth week are thin, cold, and weak, refuse the food and soon die unless supplied with another ration. All of the mice with spontaneous tumors, full fed on Sweet's food after operation, emaciated rapidly toward the end of the period of observation, their average loss of weight after thirty-five days being 30 per cent. or slightly more than that of mice underfed on the same diet.

Although the end results as regards loss of weight were nearly the same in the mice underfed and the mice full fed on Sweet's food, there were essential differences in the conditions imposed on the two groups of animals. The group which received an insufficient ration of the diet for some days prior to operation was losing weight when it was performed, whereas the mice of the other group were operated upon when still well nourished on an ordinary diet, and, being given thereafter an abundance of the special food, they lost little weight for some days. The effect of these differences is plainly to be seen in text-figures 11 and 12 which have to do with the results in the animals of the latter group. Recurrences in them (text-figure 11) took place as frequently and as soon as in the control animals (text-figure 7). Growth of the grafts (text-figure 12) on the other hand was delayed as greatly as in the underfed mice (text-figure 10).

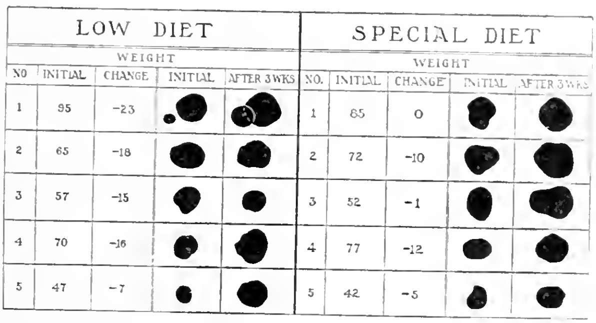
There is a simple explanation for these facts. Under ordinary conditions (text-figures 7 and 9) the appearance of a recurrence precedes by a considerable period the development of grafts,—as would naturally follow from the circumstance that the bit left for a recurrence possesses a vascularization and other connection with the tissues, whereas the grafts must acquire this, a proceeding which takes some days. During these days the mice fed on Sweet's food had begun to lose weight, and the grafts on starting to grow had the factor of general malnutrition against them. The bits left for recurrences, on the other hand, were able to begin growing almost at once, at a time when the dieting had just been started, and the animals were still well nourished.

All in all the findings show that it is possible in many cases to hinder the development of bits of spontaneous mouse tumor disseminated at operation, by feeding the animal after operation on a special diet that entails a gradual loss of weight. In order to delay

the development of recurrences from tumor bits left *in situ* a more drastic treatment is required, namely, dieting that entails loss of weight previous to the operation.

THE NATURE OF THE INFLUENCE.

In what way are these results brought about? Has Sweet's modification of Mendel's food an influence to check the growth of tumors other than that exerted through the loss of weight that it entails? There is some reason to doubt this. Several experiments made with rats carrying the Flexner-Jobling tumor show that this growth is as unaffected by a diet of Sweet's food as by underfeeding on a mixed diet (text-figure 13). But to settle the point definitely



LOW DIET					SPECIAL DIET				
	WEIGHT					WEIGHT			
NO	INITIAL	CHANGE	INITIAL	AFTER 3 WKS	NO.	INITIAL	CHANGE	INITIAL	AFTER 3 WKS
1	95	−23			1	85	0		
2	65	−18			2	72	−10		
3	57	−15			3	52	−1		
4	70	−16			4	77	−12		
5	47	−7			5	42	−5		

TEXT-FIG. 13. This shows that a diet of Sweet's food had no more effect upon the development of the Flexner-Jobling tumor than had underfeeding on a mixed diet.

tumors more sensitive to alterations in the general nutrition must be used.

The effects of the dieting can scarcely be to stimulate immunity processes in the host. For, as has been pointed out, the tumor cells are merely held in check, remaining alive and capable of rapid growth when the host is returned to ordinary food and regains its weight. In many instances, doubtless, the cells suffer directly from the general starvation of the body. There is reason to suppose, though, that most of the effect on them is indirect, through a limitation in the host's ability to form a connective tissue scaffolding and vascularization for their support. Whether the absence of this "stroma

reaction" is the essential factor in resistance to tumors, as assumed by Russell,[9] need not be here discussed. It is sufficient to know that all tumors and grafts of tumors, except those developing wholly by the invasion and replacement of normal structures, need in the course of their growth a stroma furnished by the host. In mice losing weight the proliferative activity of the host's tissues,—on which the elaboration of a stroma must ultimately depend,—is much decreased. This is shown, first by the extreme slowness with which wounds heal in these animals, and second by the delayed organization about inert, foreign bodies. I have found that clean, well apposed wounds frequently stay unhealed for a week or ten days. The encapsulation of agar-agar injected beneath the skin is also very slow.

Experiment 1.—Sixteen rats about three fourths grown were paired against one another according to weight, and half were full fed, half underfed on the same diet of mixed food. When the underfed group had begun to lose weight all were injected subcutaneously in each flank with 0.08 c.c. of 1.5 per cent. agar in Ringer's solution. The agar was cooled in the syringe, and forced through a small needle, whereby it was broken into many small fragments. It was kept from extending back along the injection track by pressure with a rubber-covered clamp. Thus each rat received in its subcutaneous tissue two discs or buttons of fragmented agar. Seven days after the injection the animals were killed and sections made through the center of each agar disc.

The reaction to agar has been often studied and need not be described further than to say that, following endothelial migration, strands of connective tissue extend among the bits of jelly which are at length wholly encapsulated and, little by little, penetrated and absorbed. These processes were found to be far advanced in the control animals of the present experiment, at a time when in the dieted ones a capsule about the agar was almost lacking and organization was just beginning (plate 21, figures 1 and 2). The experiment has been several times repeated, using both rats and mice, with the same results.

If the loss of weight caused by underfeeding retards the growth of tumors, how does it happen that tumors progress so rapidly in emaciating human beings? Probably in some instances because in

[9] Russell, B. R. G., *Third Scientific Report of the Imperial Cancer Research Fund*, 1908, 341.

man, as in mice, certain tumors are able, *ab initio,* to obtain their food with ease in a starving body. It seems likely that certain other tumors during their development gain in the ability to obtain food through the survival and proliferation of the cells most suited to cope with the increasingly difficult conditions.[10] According to this idea a tumor made up at first of a mixture of cells sensitive and insensitive to alterations in the general nutrition would, as the host emaciated, come to consist of elements little affected by the circumstance. Some experiments were performed to test the hypothesis. Transplanted tumors which had grown for some weeks in full fed hosts and hosts underfed to the point of emaciation were transferred to underfed individuals. According to the premises, the tumors from the underfed hosts should have given the better results. But no differences were noted.

SUMMARY.

Previous work has shown that the growth of grafts of transplantable tumors can be in many cases prevented or retarded by underfeeding the new host or by putting it on a special diet. The effect of such treatment on large tumors has been little studied; and the effect on metastases and recurrences has not been studied at all. Apart from certain clinical observations nothing is known as to the influence on spontaneous tumors of alterations in the diet.

Experiments with transplanted rat and mouse tumors along the lines thus suggested show that large growths of certain strains are checked in their development by underfeeding the host upon a special diet (Sweet's modification of one of Mendel and Osborne's foods) or in some cases by simple underfeeding. Two metastasizing mouse tumors are instances in point. They stopped growing or grew very slowly in hosts underfed on the special diet. The Flexner-Jobling rat carcinoma, on the other hand, was unaffected by the most rigorous underfeeding on a mixed diet when this was begun after the tumor had been growing for a short period. Experiments

[10] Loeb has shown that tumors in mice treated with colloidal copper undergo regressive changes at first, but that a strain of cells unaffected by the drug may survive, from which the tumor eventually proliferates (Loeb, L., personal communication).

to test the influence of underfeeding upon recurrences of this tumor gave results that varied from series to series of animals. The findings strongly indicate that generalizations from work with transplanted tumors as regards the effects of diet on spontaneous growths are unwarranted.

By underfeeding on Sweet's food mice with spontaneous tumors, beginning some days prior to operation, it has proved possible in most cases to delay for a relatively long period the development of recurrences and the growth of tumor bits (grafts) disseminated at the time of surgical interference. The treatment entailed great loss of weight. Tumor mice kept on ordinary diet previous to operation, but put thereafter on an abundant ration of Sweet's food, developed recurrences as early as the tumor mice on ordinary diet; whereas the growth of auto-implants was, relatively speaking, much delayed. These results seem attributable rather to a gradual malnutrition induced by the special food than to the circumstance that it lacked a growth principle. In none of the dieted mice was a definite cure obtained. Ordinarily a recurrence appeared and the grafts began to grow soon after the host, again on ordinary food, had regained weight.

A few spontaneous tumors seem absolutely unaffected by the most rigorous dieting.

Wounds heal with marked slowness in animals that have become thin as a result of dieting, and an inert foreign body (agar-agar) injected subcutaneously is very slowly encapsulated and organized. In these facts may be found a suggestion as to the method whereby dieting delays tumor growth. For it may well be that, with a lessened proliferative activity of the host tissue, the elaboration of a vascularizing and supporting stroma such as most tumors depend upon for their growth, at least indirectly, is much delayed.

The rapid growth of tumors in emaciating individuals is not incompatible with the present findings. Such growth may be consequent upon a selection in the host of those cells most fit to cope with the increasingly difficult nutritive conditions. But experiments designed to demonstrate this have been unsuccessful.

It is conceivable that recurrences of certain human tumors and

the development of metastases may be delayed or prevented for a period by methods somewhat similar to those employed against spontaneous mouse tumors in the present investigation. But generally speaking only the more malignant human tumors would require such palliative measures, and these are precisely the ones that would prove,—if experience with mice is an index,—least amenable to alterations in the nutrition of the host.

It is a pleasure to acknowledge the help of Dr. Linda Lange in this work.

EXPLANATION OF PLATE 21.

FIG. 1. Cross-section through an agar mass removed seven days after its injection into the subcutaneous tissue of a young rat which was losing weight as the result of underfeeding on a mixed diet. The encapsulation and organization are slight as compared with them in figure 2.

FIG. 2. Cross-section of an agar mass from a full fed control rat. Methylene blue and eosin.

BACTERIAL ANTIFERMENTS.

Studies on Ferment Action. XVII.*[1]

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The resistance of bacteria to enzyme action, because of its importance as a factor in the defense of the invading organism against the destructive agencies of the host, has interested several observers, among them Kruse (1), Fermi (2), Kantorowicz (3), and Weinkopff (4). Influenced possibly by the epochal advances in protein chemistry and by the fact that bacterial antigens are probably purely protein in character, workers in immunology have naturally investigated with great care the bacterial proteins, and the effect of proteolytic enzymes upon them.

The older conception that bacteria resisted digestion because of some vital property had to be discarded when it was found that organisms killed by chloroform, toluol, carbolic acid, etc., seemed to resist digestion as well as viable bacteria. It has so far been the implied idea that since bacteria contain protein, such protein should be an available substrate for enzyme action, as casein or edestin might be used; in other words, that the bacterial cell represented a naked and a freely exposed mass of protein. In dealing with an intact cell there are, however, certain factors which would indicate that this position is untenable.

Were we to assume that bacteria have a limiting membrane, such a membrane would, if an analogy to plant cells is permissible, contain no protein (Czapek (5)); if similar to animal cells they would probably be protected by a predominatingly lipoidal membrane (Meyer, Overton). There is, of course, no direct evidence, although with the exception of Hamm (6) there has been a uniformity in the reports of the absence of nitrogenous material even from the capsular material derived from the lower organisms. The absence of cellulose in bacteria is of no great import, for many of the true fungi have no cellulose wall and are protected

* Received for publication, August 7, 1914.

[1] We are under obligations to Dr. William H. Park and his associates, of the New York Board of Health, and to Dr. A. P. Hitchens, of Glenolden, Pa., who have kindly placed large amounts of diphtheria and tubercle bacilli at our disposal; and to Professor Hans Zinsser for numerous courtesies.

only by a delicate membrane; unfortunately the evidence as to the presence or absence of chitin, which might have some bearing on the subject, is conflicting.

Bacteria do, however, contain fats and lipoids in varying amounts, which, because of their marked effect on surface tension, would for purely physical reasons tend to become concentrated at the periphery of a colloidal system such as the bacterial protoplasm. With or without a morphologically distinct limiting membrane we can reasonably assume that the external surface of the bacterial cell is potentially lipoidal. Upon such a substrate we should expect lipolytic rather than proteolytic action. Immunologists have recognized that in cytolysis there are other factors than such distinctly protein manifestations of immunity as are exemplified by the precipitin and agglutinin reactions. Zinsser (7) has recently emphasized this fact. In this connection it is interesting to recall again an observation of Metchnikoff (8). Metchnikoff noted that the intestinal tract of the larva of certain insects (moths) was free from bacteria, and reasoned that this absence of intestinal flora must have some connection with the nature of the enzymes secreted into the gastro-intestinal tract. He calls attention to the fact that these insects, utilizing waxes and fats to a large extent, must secrete powerful lipases.

Oppenheimer (9), reviewing the subject of bacteriolysis from an entirely different standpoint, reaches a similar conclusion. He says: "I have formerly classified these substances (cytolysins) among the ferments because the view was prevalent that the destruction of the cell membrane was due to a specific proteolytic ferment." This view must be discarded, but it is probable that we may later place them among the lipases (10). In a recent paper (11) we have shown by microchemical analysis that bacteriolysis is not necessarily associated with proteolysis.

As briefly outlined, the failure of proteolysis by the action of proteolytic ferments on the intact cell offers then no problem in itself, for there is probably no exposed protein substrate upon which the enzyme may act.

Kantorowicz (3) has probably studied the subject most carefully. He confirmed the older observations of the absence of digestion of the intact cell whether living or dead, and showed that heating Gram-negative organisms to 70° C. made them lose their power of resistance, while Gram-positive organisms would resist digestion even after boiling. He concluded that the resistance was due to an antiferment. He found that dried organisms were as resistant as fresh bacteria, but noted that after drying, grinding in a mortar, and extracting with acetone, the organisms lost their resistance. He assumed two factors to be concerned in bacteriolysis, one of which had to do with the overcoming of the antiferment. Kruse (1) did not, however, agree with the conclusion of his pupil, and doubted that an antiferment as such was involved in the resistance of the organisms. He inclined to the view that differences in resistance among bacteria were due to differences in permeability of the organisms.

Fermi (2) working with cultures, introducing ferments into culture media, reached conclusions similar to those of Kantorowicz. He noted, however, that dried organisms were less resistant than those freshly killed.

The foregoing observations were made by using suspensions of organisms either living or killed in various ways, to which the enzyme was added and the resulting digestive effect observed by the relative clearing of the suspensions. This method is open to several objections. The interpretation of the results is inexact and arbitrary, depending on the observer's judgment, and varying under different conditions. Probably of more importance is the fact that solution of the organisms is interpreted as proteolysis. Solution of a bacterial cell may be largely a physical process related most intimately to changes in its lipoidal constituents. The actual proteolytic cleavage is a purely chemical phenomenon and cannot be determined by ocular observation. This distinction, to which Jobling and Strouse (10) have previously called attention, should be clearly kept in mind, for the careless use of these terms has led to considerable confusion in the interpretation of some immunological experiments. There is, furthermore, a technical error in that it is not possible by the means so far employed to present an approximately equal amount of substrate for the enzyme action, which is important when the comparative rate of digestion of different organisms is sought.

In order to obtain results which would be free from the objections above referred to, our observations have been based exclusively on the determination of non-coagulable nitrogen before and after digestion. These were made by means of the Folin method. Only by such a method can we determine the exact percentage of digestion. We have used organisms grown on agar in large flat bottles. The bacteria were washed, dried *in vacuo* at a low temperature, and then ground in an agate mortar. During the process of drying changes must occur in the colloidal state of the external limiting membrane, so that the uniform resistance of various bacteria to proteolytic action while intact is overcome, and the degree of resistance remaining should be an index of the actual antiferment power of the different organisms.

One other physical factor, the intimacy of the lipoid-protein combination, must be taken into consideration. Numerous workers have pointed out that the lipoids occurring in cells are so closely combined with the proteins that it is impossible by the ordinary

extraction methods to break up their union. This can be accomplished only by a complete hydrolysis of the protein. Thus the determination of bacterial lipoids by means of extraction in the Soxhlet apparatus will give results which are much too low, the real ratio of the lipoidal substances being obtained by extraction after saponification. We have noticed during the course of the work that the lipoids from the Gram-positive bacteria are proportionately extracted more slowly by means of lipoid solvents than from the Gram-negative organisms.

The bacterial emulsions were, as a rule, made up in a 1 per cent. suspension. When fresh organisms were used an effort was made to have the bacterial concentration about one milligram of nitrogen per cubic centimeter. The total non-coagulable nitrogen both before and after digestion was determined by precipitating the coagulable protein with acetic acid and salt, boiling for ten minutes, filtering through kaolinized hard filter paper, and making the usual Folin determination on the filtrate. The trypsin used was freshly prepared before each experiment by dissolving a dried preparation in physiological salt solution.

The lipoid content of the bacteria was determined by saponifying on the water-bath for two hours with alcoholic potash, acidifying, and extracting thoroughly with ether. The iodin value of the lipoids was obtained by the usual Wijs method. It should be noted, however, that the iodin determinations, while often repeated, were of necessity carried out on very small amounts of lipoids, and some caution must be observed in basing conclusions on single observations. When it was not possible to repeat the determinations, we have placed a question mark with the figure to denote the fact. Further observations which we hope to make on larger amounts of material will no doubt give more accurate values. It is reasonable to assume that the lipoid extract and the degree of unsaturation will show marked fluctuations under the varying conditions of growth, nutrition, age, and oxygenation, and, as will be noted, the variations in the total lipoid content of different growths of the same organism were at times marked. Lyons (12) has studied the influence of sugars on the fat content of bacteria, and Cramer (13) has carried out similar experiments.

The amount of lipoids (including fats) has been determined by several observers, as shown in the following table (table I). Inasmuch as the majority of these observations were made by simple ether extraction, the values given are in some instances probably much too low.

TABLE I.

Organism.	Lipoids (including fats).	Observer.
Tubercle bacilli	36–44 per cent.	Baudran (14).
Tubercle bacilli	40 per cent.	Kresling (15).
Tubercle bacilli	31.56 per cent.	Levene (16).
Tubercle bacilli	37.57 per cent.	de Schweinitz and Dorset (17).
Tubercle bacilli	26.2–28 per cent.	Hammerschlag (18).
Bacillus mallei	7.91 per cent.	de Schweinitz and Dorset (19).
Diphtheria bacillus	1.62 per cent.	Dzierzgowski and Rekowski (20).
Meningococci	5.94 per cent.	Ditthorn and Woerner (21).
Pneumobacillus	1.7 per cent.	Brieger (22).
Anthrax bacillus	7.8 per cent.	Dyrmont (23).
Bacillus prodigiosus	4.83 per cent.	Kappes (24).
Bacillus xerosis	8.06 per cent.	Kappes (24).

PROTOCOL I.

Effect of Lipoidal Extraction on the Rate of Digestion of Tubercle Bacilli.

Bacterial suspension 1 c.c.	Trypsin.	Total nitrogen.	Gross digestion.	Digestion, per cent.	Lipoid content, per cent.
Tubercle bacilli[2] (dried)............	0.2 c.c.	0.55 mg.	0.13	23	31.2
Tubercle bacilli (extracted with ether, chloroform, or alcohol)..........	0.2 c.c.	0.74 mg.	0.33	44	9
Tubercle bacilli (extracted in Soxhlet apparatus) (ether 120 hrs.; alcohol 100 hrs.; benzol 50 hrs.)..........	0.2 c.c.	0.80 mg.	0.46	57	7

The suspensions were made up with an equal amount of sodium carbonate solution.

To these we can add the following determinations:

Tubercle bacilli	32.7 per cent.
Staphylococci	4.51–8.5 per cent.
Diphtheria bacilli	5.5 –7.5 per cent.
Typhoid bacilli	7.0 –8.2 per cent.
Colon bacilli	4.2 –8.15 per cent.
Subtilis bacilli	1.7 per cent.

There are no available determinations of the iodin values of

[2] Previously killed by boiling.

these lipoids with the exception of those made upon the waxes and fats of the tubercle bacillus.

The values which we have found are as follows:

Tubercle bacilli	20 for total lipoids; 24.2 for fatty acids.
Staphylococci	60–80
Diphtheria bacilli	80–110
Typhoid bacilli	33–38
Colon bacilli	32–40
Subtilis bacilli	44 (?)
Tetanus bacilli	44 (?)

Having previously shown that the unsaturated fatty acid radical is antitryptic and its inhibition proportional to the degree of unsaturation (25); that such unsaturated lipoids can be isolated from tubercle bacilli (26) and from tuberculous caseous material (27); that serum antitrypsin consists of similar lipoids, either as free fatty acids, as esters of cholesterol, or combined with lecithin (28), we considered it reasonable to assume that bacteria might resist digestion in a degree proportional to the amount of the unsaturated lipoids contained. It will be remembered that Kantorowicz found that the antiferment was removed from the bacteria when they were ground up and extracted with acetone.

INFLUENCE OF LIPOIDAL EXTRACTION ON DIGESTIBILITY.

We first determined the influence of lipoidal extraction on the tryptic digestion of tubercle bacilli. 1 per cent. suspensions of (1) freshly dried organisms, of (2) extracted bacilli (with 9 per cent. of lipoids), and of (3) extracted bacilli (with 7 per cent. of lipoids) were used. The rate of digestion is shown in protocol I. The organisms used in this work were obtained from Dr. Hitchens and had been killed by heating at high temperatures. In addition they were old, and so some of the unsaturated fatty acids may have become oxidized.

A similar result was obtained with other organisms, as is shown in the following experiment with typhoid and colon bacilli (protocol II). Müller (29) noted that typhoid bacilli from which the lipoids had been extracted were more easily digested by leucocytes.

PROTOCOL II.

Effect of Lipoidal Extraction on the Rate of Digestion of Colon and Typhoid Bacilli.

Bacterial suspensions 1 c.c.	Total nitrogen.	Non-coagulable nitrogen.	Substrate.	Gross digestion.	Increase in non-coagulable nitrogen.	Digestion, per cent.
Colon bacilli..........	1.77 mg.	0.17 mg.	1.60 mg.	0.545 (−0.17)	0.375 mg.	23
Ether-extracted colon bacilli.............	1.87 mg.	0.23 mg.	1.64 mg.	0.95 (−0.23)	0.72 mg.	44
Typhoid bacilli........	1.08 mg.	0.14 mg.	0.94 mg.	0.66 (−0.14)	0.52 mg.	56
Ether-extracted typhoid bacilli............	1.0 mg.	0.2 mg.	0.8 mg.	0.87 (−0.2)	0.67 mg.	84

THE LIPOIDS AS ANTIFERMENTS.

We have previously noted that the lipoids extracted from tubercle bacilli were antitryptic when saponified (26). That the same is true of the lipoids from other organisms is shown in the following experiment.

Pure cultures of staphylococci and of colon bacilli were removed from bottles, washed, and saponified. After acidification, the lipoids were extracted and again saponified. The soaps were then dissolved in physiological salt solution. Various amounts were mixed with one unit of trypsin, incubated for thirty minutes, and casein was then added for digestion. In another set of tubes the unsaponified bacterial lipoids, suspended in salt solution, were used in equal amounts. The relative inhibition will be noted in protocol III.

PROTOCOL III.

Effect of Bacterial Lipoids, Saponified and Emulsified, on Tryptic Digestion.

Tube No.	Casein.	Trypsin.	Staphylococci lipoids saponified.	Colon lipoids saponified.	Staphylococci lipoids emulsified.	Colon lipoids emulsified.	Gross casein digestion.	Digestion, per cent.
1	2 c.c.	0	0	0	0	0	0.0 mg.	0
2	2 c.c.	0.1 c.c.	0	0	0	0	1.66 mg.	100
3	2 c.c.	0.1 c.c.	2 c.c.	0	0	0	0.217 mg.	13
4	2 c.c.	0.1 c.c.	0	2 c.c.	0	0	0.715 mg.	43
5	2 c.c.	0.1 c.c.	0	0	2 c.c.	0	1.33 mg.	80
6	2 c.c.	0.1 c.c.	0	0	0	2 c.c.	1.54 mg.	92

The iodin value of the lipoids was 97 for those from the staphylococci, and 32 from the colon bacilli.

THE COMPARATIVE RATE OF DIGESTION.

We next studied the comparative rate of digestion of bacteria, and the relation to the lipoids contained in the bacteria. Such an experiment is shown in protocol IV, and has for convenience been charted (text-figure 1). As a lipoidal antitryptic index we may multiply the percentage of lipoids by the iodin value, as determined for the organisms used in this experiment.

PROTOCOL IV.

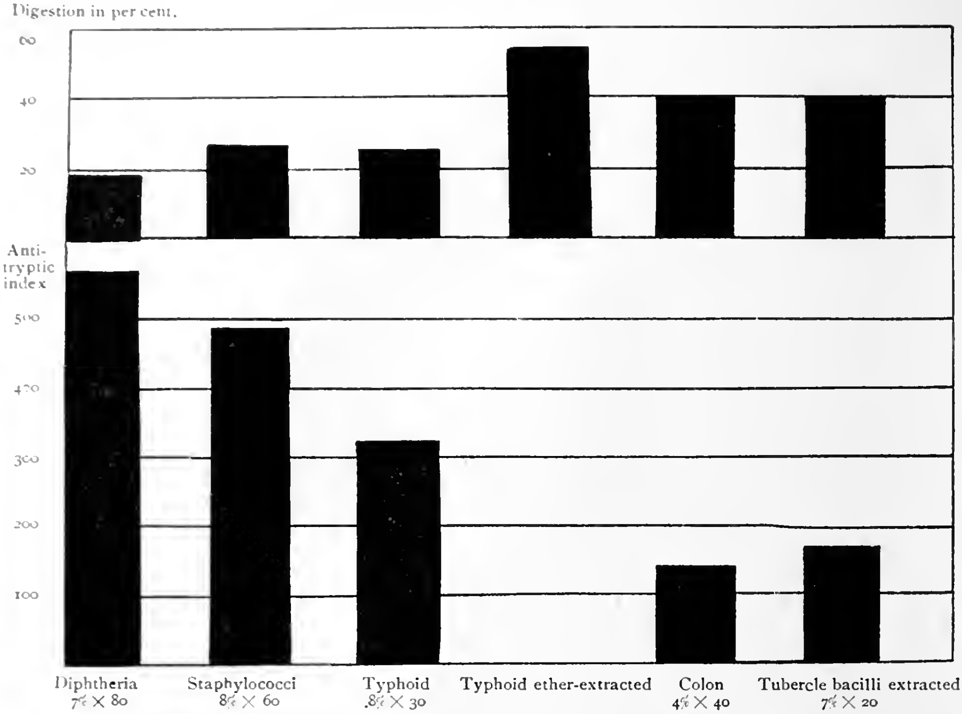
Comparative Rate of Tryptic Digestion of Bacteria.

Bacterial suspension 1 c.c.	Total nitrogen.	Non-coagulable nitrogen.	Substrate.	Gross digestion.	Increase in non-coagulable nitrogen.	Digestion, per cent.
Diphtheria bacilli	1.0 mg.	0.12 mg.	0.88 mg.	0.28(−0.12)	0.16 mg.	18
Staphylococci	0.83 mg.	0.15 mg.	0.68 mg.	0.31(−0.15)	0.16 mg.	23
Typhoid bacilli	1.42 mg.	0.21 mg.	1.21 mg	0.5 (−0.21)	0.29 mg.	24
Typhoid bacilli (ether-extracted)	0.9 mg.	0.35 mg.	0.55 mg.	0.66(−0.35)	0.31 mg.	56
Tubercle bacilli (ether-extracted)	0.83 mg.	0.12 mg.	0.71 mg.	0.4 (−0.12)	0.28 mg.	40
Colon bacilli	0.8 mg.	0.12 mg.	0.68 mg.	0.39(−0.12)	0.27 mg.	40

Diphtheria bacilli .... 7 per cent. × 80 = 560
Staphylococci .... 8 per cent. × 60 = 480
Typhoid bacilli .... 8 per cent. × 30 = 240
Typhoid bacilli (extracted) .... ? ? ?
Colon bacilli .... 4 per cent. × 40 = 160
Tubercle bacilli (extracted) .... 7 per cent. × 20 = 140

This offers, of course, only a general approximation, and cannot take into consideration the fact that the lipoids vary no doubt in the intimacy of their connection with the proteins, a factor which must influence their ability to protect the protein. The digestion as determined was as follows:

Diphtheria bacilli .... 18 per cent.
Staphylococci .... 23 per cent.
Typhoid bacilli .... 24 per cent.
Typhoid bacilli (extracted) .... 56 per cent.
Colon bacilli .... 40 per cent.
Tubercle bacilli (extracted) .... 40 per cent.



TEXT-FIG. 1. Relation of the antiferment index to the rate of digestion of various organisms.

These figures show a striking parallelism between the antitryptic property and the lipoidal protecting bodies.

A similar experiment can be shown if we compare the rate of digestion of the Gram-positive organisms, the lipoids being as follows:

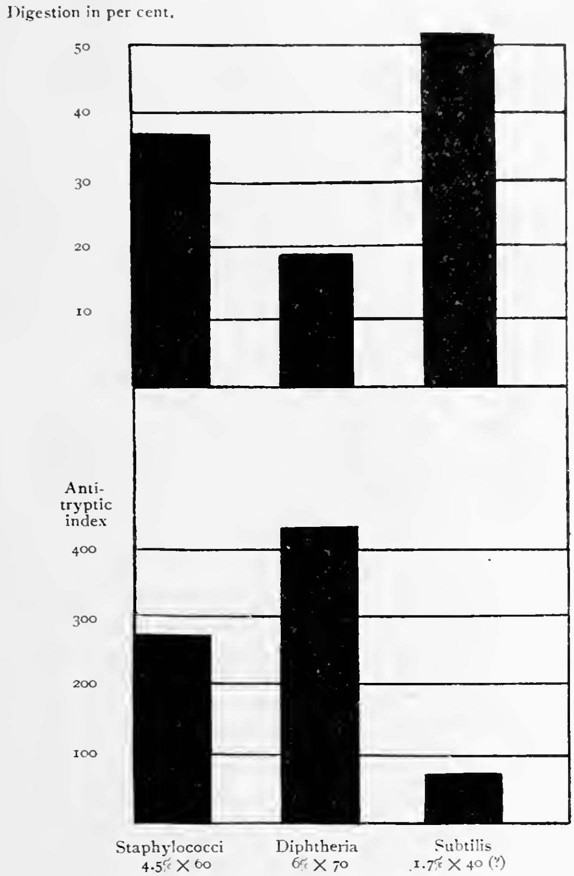
Diphtheria bacilli .... 6.0 per cent. × 70 = 420
Staphylococci .... 4.5 per cent. × 60 = 270
Subtilis bacilli .... 1.7 per cent. × 40 = 68

PROTOCOL V.

Comparative Rate of Digestion of Staphylococci and Diphtheria and Subtilis Bacilli.

Bacterial suspension 2 c.c.	Total nitrogen.	Non-coagulable nitrogen.	Substrate.	Gross digestion.	Increase in non-coagulable nitrogen.	Digestion, per cent.
Staphylococci.........	1.54 mg.	0.375 mg.	1.17 mg.	0.8 (−0.375)	0.425	36
Diphtheria bacilli.....	1.66 mg.	0.19 mg.	1.47 mg.	0.47 (−0.19)	0.27	19
Subtilis bacilli........	1.3 mg.	0.48 mg.	0.82 mg.	0.909 (−0.48)	0.429	52

The organisms digested in the order indicated, the digestion being least with the diphtheria bacilli. The results are shown in protocol V and in text-figure 2.



TEXT-FIG. 2. Relation of the antiferment index to the rate of digestion of Gram-positive organisms.

THE EFFECT OF HEAT.

It has been mentioned that a difference existed in the resistance to heat of the Gram-positive and the Gram-negative organisms. This is shown in the following experiments.

A suspension of typhoid bacilli was heated at 50°, 60°, 70°, and 100° C. The

tubes were then subjected to the action of trypsin. The percentage of digestion was as follows:

50° C. = 31 per cent.
60° C. = 31 per cent.
70° C. = 36 per cent.
100° C. = 41 per cent.

As Kantorowicz (3) has found, there is a reduction of the resistance after heating to 70° C., but the effect is not so striking as one might expect if a complete destruction of the antiferment had resulted. The Gram-positive organisms, staphylococci for example, show no change after heating, unless it is a slight increase in the resistance. The percentage of digestion was as follows:

Unheated = 55 per cent.
50° C. = 54 per cent.
60° C. = 35 per cent.
70° C. = 41 per cent.
100° C. = 38 per cent.

In a second experiment the results were as follows:

Unheated organisms = 16 per cent.
70° C. = 18 per cent.
100° C. = 14 per cent.

LIPOIDAL ADSORPTION BY BACTERIA.

We have previously mentioned that bacteria suspended in normal serum are able to adsorb the unsaturated lipoids from the serum, and by virtue thereof to become more resistant to the action of trypsin (30). Such an experiment follows.

A suspension of typhoid bacilli was incubated with 10 c.c. of normal guinea pig serum for six hours. The bacteria were then centrifuged, washed, and dried at a low temperature. Untreated bacteria were used as a control. Emulsions were prepared containing 1 mg. of nitrogen per cubic centimeter, and to each was added 0.1 c.c. of trypsin solution and 2 c.c. of a 0.5 per cent. solution of sodium carbonate. Digestion was permitted for six hours. The digestion of the untreated bacteria was 75 per cent., of the serum-treated bacteria 23 per cent.

The lipoidal adsorption can be shown in a more direct manner, as is illustrated in the following experiment.

Dried subtilis bacilli were added to 25 c.c. of horse serum and incubated for three hours. The bacteria were then washed after being centrifuged from the serum. The lipoid content of the bacteria and of the supernatant serum was now determined.

Total lipoids in 25 c.c. of original serum		0.083	gm.
Total lipoids in 25 c.c. after incubating with bacteria		0.060	gm.
		0.023	gm. loss.
Weight of bacteria recovered	0.6 gm.		
Lipoid content originally present, 1.7 per cent	0.0102 gm.		
Total lipoids recovered from bacteria		0.036	gm.
Less lipoids originally present		0.0102	gm.
		0.0258	gm. gain.

While there has been a loss of 0.023 of a gram in the ether-soluble substances from the serum, the bacteria show a corresponding gain. The absorption of lipoids is made especially evident in this case because of the small lipoid content of the organisms used. The lipoid determinations were made after thorough saponification of both bacteria and serum on the water-bath for three hours.

DISCUSSION.

Inasmuch as the bacterial lipoids may show more marked fluctuations than the other constituents, the quantity depending largely on the composition of the nutrient media and to some extent on the age of the culture, and the chemical quality, unsaturation, being subject to changing oxidative conditions, we are of the opinion that the demonstration that the antiferment property of bacteria depends on definite quantitative as well as qualitative differences in the lipoidal constituents of the bacteria may offer the means of solution of some still obscure problems in immunity. Thus in the study of virulence, depending on the invasive power of the organisms and on their resistance against the defensive mechanism of the host a partial solution might be found in a comparative study of the lipoids under different conditions of bacterial existence. We have experiments under way to elucidate this point. So too, a further study of the adsorption of the lipoids from the serum offers an attractive field. While in this process the resistance of the intact bacteria to proteolytic enzymes is not increased, for on such organisms the

enzymes probably find no substrate upon which they may become fixed and active, nevertheless the metabolic processes of the cell, depending wholly upon the rate of exchange of nutritive and excretory products through the cell membrane, must be profoundly altered. Indeed it might be conceived that such a disturbance of the proper functioning of the membrane would under some conditions lead to the death of the cell. On the other hand, an absorption of serum lipoids, by preventing the excretion of toxic metabolic products, such as leucocydins, virulins, etc., might bring about phagocytosis of organisms otherwise resistant. That the so called opsonins are closely related to the lipoids seems to have been established by the work of Stuber (31).

That the bacterial lipoids influence the intracellular proteolytic activity becomes apparent when we compare the amount of non-coagulable nitrogen contained in various organisms. Thus diphtheria bacilli, with a high antiferment index, have only from 5 to 7 per cent. of the nitrogenous material in a non-coagulable form, whereas in subtilis bacilli, with a low antiferment index, a correspondingly greater protease activity is indicated by the presence of from 33 to 35 per cent. of the total nitrogen in a non-coagulable form. Staphylococci, typhoid bacilli, and colon bacilli occupy an intermediate position. This influence is analogous to the effect of the antiferment on the protein metabolism of higher organisms, as we shall show in a subsequent paper.

The antiferment, being non-specific, is able not only to inhibit the action of the digestive ferments, but is probably the agent concerned in preventing excessive protease action, or autolysis, in the cells. It is easy to understand that with the death of the cells and subsequent development of an acidity because of the failure in the removal of acid waste products, the antiferment property is greatly lessened by changes in the state of dispersion brought about by the increase in acidity. This lowering of the antiferment property of the unsaturated lipoids by a change in their dispersion is most easily demonstrated with serum antitrypsin. The morphological expression of the process is possibly to be found in the development of the so called myelin figures found in cells stained during the early stages of autolysis. It is probable that the increase in the rate of

autolysis following the addition of certain inorganic colloids to liver emulsions, noted by Ascoli and Izar (32), depends on an absorption of the lipoidal antiferments.

Kantorowicz (3), in view of the difference in behavior of the antiferment in Gram-negative and Gram-positive organisms, is inclined to the view that the antiferments are in this case different. It seems more reasonable to assume that the difference noted is due to a purely physical condition. It is possible that in the Gram-positive organisms the lipoids are in more intimate association with the protein molecule, so that when dispersion changes are brought about, as by heating, the relative protection by these lipoids remained unaltered. This idea would find support in view of the fact that the lipoids from Gram-positive organisms resist extraction by lipoid solvents to a greater extent than do those from Gram-negative bacteria.

In view of the work of Tamura (33), definitely showing that the bacterial constituent responsible for the Gram stain is lipoidal in that it can be found in the lipoid extract, and as the unsaturated fatty acids and their salts take the Gram stain easily, because the iodin can be bound to the unsaturated carbon atoms, it would seem rational to seek some connection between the degree of unsaturation of the bacterial lipoids and the Gram staining ability. From this point of view the high iodin values of the lipoids from diphtheria bacilli and staphylococci, as compared with those of typhoid or colon bacilli, might seem significant, were it not for the fact that in the case of the tubercle and subtilis bacilli, the results are apparently the reverse. Knudson (34), working in Gies's laboratory, finds, however, that the lipoids from tubercle bacilli, which have a low iodin value (24), contain several of the higher unsaturated fatty acids.

As a result of our study we are inclined to emphasize the importance of the unsaturated lipoids as representing probably a most important factor in the regulation of the proteolytic activity of the cellular metabolism, due to the fact that the protein lipoid combination resists the action of the proteolytic enzymes.

CONCLUSIONS.

1. Intact bacteria probably resist tryptic digestion because of the absence of an exposed protein substrate.

2. Dried organisms resist digestion in a degree proportional to their content of unsaturated lipoids.

3. Lipoidal extractives reduce the resistance to tryptic digestion.

4. The extracted lipoids (saponified) are antitryptic in a degree proportional to their unsaturation.

5. The inactivation of the antiferment in Gram-negative organisms is probably due to changes in the degree of lipoidal dispersion.

6. Bacteria adsorb lipoids from the serum when incubated at 37° C. Such organisms when dried are found to be more resistant to tryptic digestion than untreated organisms.

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LIPOIDS AS INHIBITORS OF ANAPHYLACTIC SHOCK.

STUDIES ON FERMENT ACTION. XVIII.*

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The refractory period following anaphylactic shock, noted early in the study of anaphylaxis, has been variously explained. Among immunologists the idea seems to be generally accepted that the condition is due to an exhaustion of the specific immune substance which together with complement is supposed to act upon the introduced antigen.

Rusznjak (1) first noted that immediately following anaphylactic shock, especially if protracted or following an extended latent period, a definite rise in the antitryptic titer of the animal's serum occurred. Rusznjak, assuming that the hypothesis of Rosenthal (2) in regard to the nature of serum antitrypsin was correct, interpreted his experiment as a demonstration of the splitting of proteins during anaphylactic shock. He furthermore advanced the idea that the period of resistance following shock was due to this increase in the antiferment property of the blood. Seligmann (3) could not confirm these findings, but Pfeiffer and Jarisch (4) later found the observations to be correct, and furthermore showed that a similar rise in antitrypsin occurred following various protein intoxications,—hemolysins, protein split products, etc. Zinsser (5) has recently shown that an increased resistance is found to anaphylatoxins following a first sublethal injection, and we have noted a similar condition with serotoxin (6). Following a sublethal serotoxin injection a well marked rise in the antitryptic titer is observed.

The observation of Rusznjak is therefore true not only for anaphylaxis but probably for every intoxication accompanied by cellular destruction. His conclusions, however, being based on the erroneous theory of Rosenthal as to the nature of serum antitrypsin, are incorrect. We have recently demonstrated that the unsaturated lipoids of the serum are the substances upon which the antitryptic property depends (7). The increase in antitrypsin observed in these cases cannot be due directly to the protein split products, but is to be explained by

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the liberation of lipoids following cellular destruction The observations of Rusznjak and of Pfeiffer and Jarisch offer then no direct evidence that protein splitting occurs during anaphylactic shock, but merely indicate that a general cellular intoxication has occurred.

The increase in antiferment might nevertheless explain the increased resistance to a second injection of antigen in a sensitized animal. This question can find a solution only if it is possible to increase the antiferment in the blood serum by some means other than a protein intoxication, for following such injury the objection can be made that there is a reduction or exhaustion of both specific and non-specific proteases or antibodies responsible for the shock, together with a destruction of complement. That complement, however, can have no relation to the phenomena, if we regard the intoxication as purely protein, is evident from the fact that the complementary action is not proteolytic but probably lipolytic, a subject which we have briefly discussed in a previous paper (8). Serum antitrypsin being lipoidal and capable of isolation from the serum by means of lipoidal extractives should on reinjection into experimental animals cause an increase in the antiferment property of the blood serum. Such an experiment can be made as follows.

METHODS OF INCREASING THE ANTIFERMENT IN THE BLOOD.

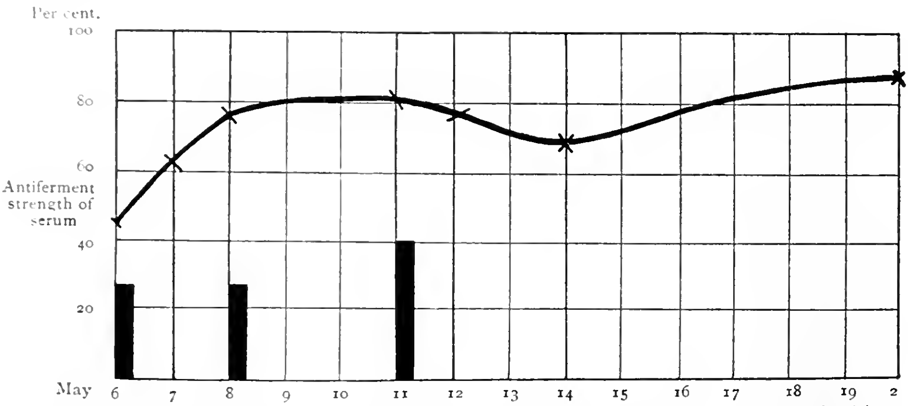
About 300 milligrams of serum lipoids extracted at various times from human and guinea pig serum were dissolved in six cubic centimeters of olive oil. Of this solution a large guinea pig received subcutaneous injections as follows:

May 6, 1914.	1.5 c.c.
May 8, 1914.	1.5 c.c.
May 11, 1914.	2.0 c.c.

The antiferment index, determined by the method discussed in our former papers, is shown in text-figure 1, in which the inhibition of tryptic digestion by 0.05 of a cubic centimeter of serum both before and after the injections is outlined. It will be noted that there is a distinct and prolonged rise from the original 45 per cent. inhibition to more than 80 per cent. as determined on May 11.

Olive oil injected subcutaneously in equal amounts had no effect on the antitryptic index.

Inasmuch as it is difficult to secure large amounts of serum lipoids we have substituted the fats prepared from egg yolk which contain large quantities of unsaturated lipoids. These were obtained as follows: The yolk was extracted twice with alcohol and twice with ether. The extracts were combined and evaporated to dryness. The mass was then extracted with ether and precipitated with acetone. Only the acetone-soluble lipoids were used. As so obtained the lipoids are semifluid and can be injected without the



TEXT-FIG. 1. Increase in antitrypsin following injection of serum lipoids subcutaneously.

use of a solvent. The increase in antitrypsin following the subcutaneous injection of one cubic centimeter in a guinea pig is shown as follows:

	Inhibition by serum before injection. June 29, 1914.	After injection. July 3, 1914.
0.10 c.c. serum	69 per cent. inhibition	79 per cent.
0.075 c.c. serum	44 per cent. inhibition	70 per cent.
0.05 c.c. serum	10 per cent. inhibition	70 per cent.
0.025 c.c. serum	0 per cent. inhibition	0 per cent.

The guinea pig was of medium weight. It was bled from the heart (two cubic centimeters) on June 29, and the egg fat injected the same day. The rise in antiferment is marked.

THE INHIBITION OF ANAPHYLACTIC SHOCK.

Having established the fact that these lipoids will, when subcutaneously absorbed, cause an increase in antiferment, we investigated the effect of such injections on the reaction of sensitized guinea pigs when the specific antigen was injected intravenously.

Six guinea pigs were sensitized on May 16, 1914, with 0.1 c.c. of horse serum intraperitoneally. Three of the animals received subcutaneous injections of serum antitrypsin dissolved in olive oil. The injections, 1 c.c. each, were made on May 21, May 26, and June 5. Purified horse serum albumen solution (1 per cent.) was used for reinjection. The toxic dose of this was determined on the three control animals and resulted as follows:

Animal No.	Weight	Dose.	Dose per gm. weight.	Result.
1	240 gm.	0.8 c.c.	0.0033 c.c.	Convulsions, respiratory spasms. Final recovery in 10 min.
2	280 gm.	1.4 c.c.	0.005 c.c.	Death in 2 min. Typical.
3	300 gm.	1.5 c.c.	0.005 c.c.	Same.

The lethal dose, 0.005 c.c. per gram weight, was now injected into the antitrypsin guinea pigs.

Animal No.	Weight.	Dose.	Dose per gm. weight.	Remarks.
4	210 gm.	1.0 c.c.	0.0047 c.c.	Scratches. No convulsions, no respiratory spasms. Remained well.
5	300 gm.	1.5 c.c.	0.005 c.c.	Death in 3 min. Typical.
6	220 gm.	1.1 c.c.	0.005 c.c.	No symptoms except restlessness. Remained well.

In two of these guinea pigs the acute shock had been completely averted. A similar and even more striking effect was obtained with guinea pigs treated with egg fats.

Ten guinea pigs were sensitized with horse serum on July 1, 1914. Six of the animals were injected previously with 1 c.c. of egg fat, on June 29. One of the animals was killed on July 3 and the antitryptic index determined, the result being shown on page 470. They received further injections of 1 c.c. each on July 10 and 16. The effect of the reinjection of a 1 per cent. horse serum albumen solution is shown in the following table.

CONTROL ANIMALS.

Animal No.	Weight.	Dose.	Dose per gm. weight.	Result.
1	170 gm.	0.425 c.c.	0.0025 c.c.	No effect.
2	230 gm.	1.15 c.c.	0.005 c.c.	Marked respiratory convulsions and spasms. Recovered in 30 min.
3	295 gm.	2.95 c.c.	0.01 c.c.	Death in 2 min. Typical.
4	275 gm.	2.75 c.c.	0.01 c.c.	Death in 2 min. Typical.

The minimum lethal dose was therefore 0.01 of a cubic centimeter per gram weight of guinea pig. The injection into the animals treated with egg fat resulted as follows:

Animal No.	Weight.	Dose.	Dose per gm. weight.	Result.
5	360 gm.	3.6 c.c.	0.01 c.c.	Immediate respiratory spasms; gradual recovery. Well after 10 min.
6	290 gm.	2.9 c.c.	0.01 c.c.	Scratched; no other symptoms.
7	235 gm.	2.35 c.c.	0.01 c.c.	No symptoms.
8	190 gm.	2.37 c.c.	0.0125 c.c.	No symptoms.
9	240 gm.	4.8 c.c.	0.02 c.c.	Marked respiratory spasms, with dyspnea for 20 min. Complete recovery.

It will be observed that all the animals receiving from one to two times the lethal dose made a complete recovery, and in three of the guinea pigs no symptoms of note were observed. The animals remained well during the next two weeks while under observation. There can then be no question but that the increase in antiferment is able to protect the animal from at least twice the minimum lethal dose of antigen and that this increase in antiferment following a protein shock must have a large share in the resistance to a second injection.

THE INFLUENCE OF LIPOIDS PRESENT IN THE ANTIGEN ON ANAPHYLACTIC SHOCK.

It has been found difficult to induce acute anaphylactic shock by means of whole bacteria, and differences have been noted in the period of time following a second injection of various antigens before symptoms of shock would be elicited, the latent period varying from one to fifteen minutes. A long latent period is common with egg albumen; indeed, Rusznjak employed egg albumen for this reason in his experiments. Inasmuch as we have recently demonstrated (9) that bacteria contain unsaturated lipoids which represent the antiferment and that they resist digestion in a degree proportional to the amount of the lipoids present; and since egg albumen resists digestion by means of the ordinary tryptic ferment unless first acted upon by pepsin in an acid medium, whereby the antiferment is destroyed because of alteration in the colloidal dis-

persion brought about by the change in reaction, we next examined the relative toxicity of an antigen before and after chloroform extraction. For this purpose we used the same preparation of purified horse serum albumen, the method of preparation of which we have discussed previously (6).

Fifty cubic centimeters of a 1 per cent. solution were prepared, of which twenty-five cubic centimeters were thoroughly shaken with chloroform and incubated for forty-eight hours, the flask being shaken at intervals. No autolysis occurred during this time. Before use the chloroform was freed from the serum albumen solution by centrifugation and filtration through a coarse paper filter. Guinea pigs were sensitized with horse serum intraperitoneally on April 13, 1914. The minimum lethal dose of the original horse serum albumen solution was determined as follows:

May 20, 1914.

Animal No.	Weight.	Dose.	Dose per gm. weight.	Result.
1	300 gm.	3.0 c.c.	0.01 c.c.	Typical anaphylactic shock. Death in 2 min.
2	300 gm.	1.5 c.c.	0.005 c.c.	Same.
3	360 gm.	0.9 c.c.	0.0025 c.c.	Recovered.
4	310 gm.	0.77 c.c.	0.0025 c.c.	Typical death in 3 min.
5	270 gm.	0.32 c.c.	0.0012 c.c.	Scratched; no other symptoms.

The minimum lethal dose was therefore about 0.0025 of a cubic centimeter per gram weight. The extracted serum albumen solution was toxic in a much smaller dose, as will be seen from the following table.

Animal No.	Weight.	Dose.	Dose per gm. weight.	Result.
6	290 gm.	0.72 c.c.	0.0025 c.c.	Death immediate and typical.
7	290 gm.	0.36 c.c.	0.0012 c.c.	Death immediate and typical.
8	320 gm.	0.2 c.c.	0.0006 c.c.	Death immediate and typical.
9	300 gm.	0.1 c.c.	0.0003 c.c.	Marked convulsions; respiratory spasms. Final recovery.

This experiment would indicate that the lipoids combined with the protein antigen may exert a considerable influence on the relative toxicity of the anaphylactic antigen, and probably explains

the difficulty encountered in obtaining uniform results with such antigens as bacteria which are relatively well protected by their lipoidal components.

THE PREVENTION OF ANAPHYLACTIC SHOCK BY THE SIMULTANEOUS INJECTION OF SOAP SOLUTIONS.

If by extraction of a lipoid containing protein antigen it becomes more toxic, it would seem reasonable that by adding the extracted lipoids to the same the toxicity should be decreased, or, if added in sufficient amount, completely neutralized. We have shown that this can actually be done with serotoxin (6), and we discussed the reasons for the use of soaps in place of lipoid suspensions. A difficulty is encountered, though, in the use of soaps, in that they will in themselves on injection cause a shock similar to that observed in anaphylaxis, so that great care must be used in working with doses that are sublethal (10). While such soaps, oleates for example, are highly antitryptic, we have noted (7) that when incubated with serum, instead of increasing the antiferment index as might be expected, they actually cause a lowering of the antitryptic titer, possibly because of solution of the serum antiferment in the soap solution. Nevertheless, when added to the anaphylactic antigen, soap solutions are able to render the reinjection harmless within certain limits, as will be observed in the following protocols. We may state, however, that we have not been able to secure these results when whole serum was used as an antigen; the results have been obtained only when a solution of horse serum albumen was used. Whether this depends on the greater stability of the albumens as contrasted with the globulins, as might be indicated in the recent work of de Waele (11), or whether it is due to the fact that the soaps lower the antiferment index when added to the whole serum, in which case the injected antigen would have less protection than before, we cannot state.

Sensitized animals and antigen solutions, the toxic doses of which have been described on page 473, were used. 1.5 cubic centimeters of the original serum albumen solution were mixed with one cubic centimeter of a 1 per cent. solution of sodium oleate and incubated

for ten minutes. The surely toxic dose of the antigen was 0.005 of a cubic centimeter per gram weight.

Weight of animal.	Dose.	Dose per gm. weight.	Result.
300 gm.	1.5 c.c. 1.0 c.c. soap solution	0.005 c.c.	No symptoms.

Similar mixtures were made with the lipoid-extracted serum albumen solution, the lethal dose of which was 0.0006 of a cubic centimeter per gram weight.

Weight of animal.	Dose.	Dose per gm. weight.	Result.
280 gm.	0.2 c.c. 1.0 c.c. soap solution	0.0007 c.c.	No effect.
260 gm.	0.4 c.c. 1.0 c.c. soap solution	0.0015 c.c.	No effect.
260 gm.	1.0 c.c. 1.0 c.c. soap solution	0.0038 c.c.	No effect.

These experiments are simply isolated examples from numerous trials which have always shown the same result. In the last protocol the protection is observed to be ample against five times the minimum lethal dose.

Whether or not this protection by the soaps is a mechanical one, due to the formation of a thin soap membrane about the aggregates of the antigen, whether it is due to the actual antiferment property of the soap, or whether its action depends on changes induced in the cellular membranes of the animal so injected, rendering the cells less permeable to the toxic substances responsible for the symptom-complex, can, of course, not be decided from these experiments. We are, however, inclined to assume that the latter is the explanation, especially in view of the work of Schultz (12), Dale (13), Weil (14), and Coca (15), showing that the origin of the shock is probably cellular and not humoral.

CONCLUSIONS.

1. The antitryptic titer of the serum can be increased by subcutaneous injections of serum lipoids (antitrypsin) and of the lipoids from egg yolk.

2. Animals so injected show a relative immunity to acute anaphylactic shock (two minimum lethal doses).

3. Extraction of lipoids contained in antigens increases the toxicity of the antigen when injected into a sensitized animal.

4. Sublethal doses of soap solutions injected simultaneously with the antigen (purified horse serum albumen) prevent anaphylactic shock.

5. The refractory state following anaphylactic shock is related in part to an increase in the antitryptic titer of the serum.

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FURTHER EXPERIMENTS UPON THE EFFECTS OF EXTIRPATION OF THE THYMUS IN RATS, WITH SPECIAL REFERENCE TO THE ALLEGED PRODUCTION OF RACHITIC LESIONS.*

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PLATES 22 TO 25.

In a previous paper (1) dealing with the effects of extirpation of the thymus of white rats, the writer reached the conclusion that total removal of the gland at an early age was not necessarily followed by modifications of the normal development, nor by characteristic changes in the skeletal system.

While this article was in press, there appeared a short paper by Klose (2) embodying results at variance with these observations. Klose described briefly the results of thymus extirpations in pigs, goats, rats, and chickens. The rats were operated upon on the fourteenth day after birth. Some of the animals showed no results from the operation, and this failure is ascribed by Klose to the presence of accessory tissue within the thyroid gland. In rats without such accessory thymus tissue, death followed after eight to ten weeks, with symptoms of cachexia thymopriva. The bones in these rats showed severe rachitic changes. Most striking were the changes in the ribs, in which there was found a typical rosary, such as is seen in human rickets. Microscopically there was an extreme widening of the zone of growing cartilage, failure of calcification in the preparatory zone, great irregularity and distortion of the cartilage columns, and their penetration by blood vessels. Klose pictures further a dense spongiosa composed in the region of the costochondral junction almost wholly of osteoid, and an accompanying transformation of the marrow into "*Fasermark*." Similar but less striking changes were found also in the femora.

Magnini (3) has also published an article dealing with the effect of thymus extirpation upon white rats. Young rats, less than 60 or 65 gm. in weight, showed effects noticeable within a few days after the operation. There developed a progressive cachexia which terminated fatally after forty to fifty days. Older rats, up to 70 gm. in weight, survived for about two months, whereas still larger animals recovered and remained alive. By the injection of suitable doses

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of thymus extract, it was possible to prevent the ill effects of the thymus extirpation. The spleen was found enlarged in 75 per cent. of the thymectomized rats, but no microscopic changes were observed in liver, adrenals, hypophysis, or testicles. No mention is made of alterations in the skeletal system.

In view of such conflicting observations, it seems desirable to bring forward additional evidence in support of the conclusions reached in the writer's previous paper. It has been suggested[1] that negative results might be due to unrecognized lobules of accessory thymic tissue which had undergone delymphoidization or other involutional change making their identification difficult. While it is hard to refute this criticism absolutely, it may be said that in those cases in which accessory tissue has been present, there has been no difficulty in recognizing it as such and in differentiating it from lymphoid tissue. Further, the failure of such minute thymic rests to undergo hyperplasia in itself suggests that they are of no great functional significance.

As regards the alleged production of rachitic changes, it was pointed out that Klose, although aware of the occurrence of spontaneous rachitis in rats, had given no data by which one could exclude this disease as a possible explanation of his findings. At that time I had not met with rachitic lesions in rats. During the past spring, however, a number of rats have been studied which showed striking changes in the bones and teeth. Among these were rats which had been thymectomized at an early age, control rats of the same litters, and rats from the stock cages. It is believed that a detailed description of some of these animals will support the view that these alterations of the skeletal system are not due to the lack of thymus secretion, but are the result of the spontaneous disease first described by Morpurgo.

This paper, therefore, includes observations (1) upon litters in which complete thymectomy was not followed by alterations in growth, nutrition, or skeletal system; (2) upon litters in which both operated animals and controls showed lesions of the bones and teeth; and (3) upon unoperated animals from the stock cages, showing similar lesions. Through the coöperation of Professor W. J. Gies, calcium determinations of the bones and teeth were made in

[1] Personal communication from Professor J. Salkind.

a number of the rats, and the results of these determinations are published in a separate paper (4).

The operative technique has been fully described in the previous study. The completeness of the operation and the possible presence of accessory thymic tissue in the thymectomized rats was controlled in each case by an examination of serial sections of the entire neck organs, including the thyroid, and the upper half of the thoracic organs. The teeth and bones were decalcified in 5 per cent. nitric acid. The staining methods included Morpurgo's modification of Schmorl's thionin-picric acid method, which differentiates even in completely decalcified specimens between osteoid and completely calcified bone.

PROTOCOLS.

Litter U.—This litter consisted of seven rats, four of which were thymectomized on July 28, 1913. During the following weeks mother and young received protective inoculations of a *B. enteritidis* vaccine, since fatal infections with this organism were prevalent at that time.

Rat U2.—Emaciated, cyanotic, and anemic; died eight weeks after operation, having gained only 10 gm. It was eaten by the other rats and could not be examined.

Rat U7.—Control. Gained poorly, remained weak, and had a bloody discharge about the eyes and nose. It was killed sixty-eight days after operation. Weight at death 38 gm. No gross or microscopic lesions were found to explain the inanition.

Rat U4.—Thymectomy. This rat also failed to gain weight normally, but remained lively, and aside from poor nutrition, showed no evidences of illness. It was killed sixty-eight days after operation, rat U7 serving as control. Weight at death 34 gm.

Autopsy.—Negative, aside from emaciation.

Microscopic Examination.—Numerous necroses, old and recent, in the liver. There was absence of spermatogenesis. Serial sections of the neck organs showed three lobules of thymic tissue lying in front of the trachea. There was also a small accessory thymus lobule, below and external to the lower pole of the left thyroid lobe.

The remaining rats of the litter (U1, U3, U5, and U6) were kept under observation until January 28 and 29, 1914, when they were killed with ether.

Rat U1.—Thymectomy. On Dec. 6 a small piece of thymus from a rat of another litter (Z4) was implanted in the peritoneal cavity. No symptoms followed this operation, and no vestiges of the implanted fragment were found at autopsy.

Autopsy.—No gross visceral or skeletal changes.

Microscopic Examination.—A few small necrotic areas are seen in the liver,

with accumulations of wandering cells in the portal spaces. Splenic follicles are large, with a broad zone of paler mononuclear cells at the periphery. There are myeloid foci in the pulp, and numerous megakaryocytes. There is also one small collection of polynuclears. Near a lymph node in the region of the salivary gland, there is a small lobule of tissue, in part necrotic, in part composed of pale epithelioid cells. This can not be identified as thymus. Adherent to the pericardium there is a lobule of tissue measuring 500 by 150 by 160 microns, which is composed of oval or fusiform cells with pale nuclei, arranged about capillaries or irregularly distributed and infiltrated with lymphoid and plasma cells. It can not be identified as thymus tissue, since it shows no Hassall bodies nor larger epithelial cell complexes, and is probably granulation tissue.

Rat U3.—Thymectomy.

Autopsy.—No gross lesions in the viscera or skeletal system, save that there are several parasitic (?) cysts of the mesentery.

Microscopic Examination.—There are no infective lesions of the liver or spleen. The series of the neck organs is incomplete in that the upper and posterior portions are torn in places. The mediastinal structures are, however, intact. No thymus tissue is present, and no accessory glands are found.

Rat U5.—Control. There are suppurative foci in the middle lobe of the right lung. The spleen is enlarged. There are no other gross lesions.

Microscopic Examination.—No infective lesions in liver or spleen.

Rat U6.—Control.

Autopsy and Microscopic Examination.—Normal organs. There is active spermatogenesis.

The weights of the viscera and other data are summarized in the following table.

TABLE I.

Rat.	Duration of experiment.	Weight at operation.	Weight at death.	Length.	Weight of spleen.	Weight of testes.	Spermatogenesis.	Weight of adrenals.	Weight of thymus.	Remarks.
U1	185 dys.	14 gm.	97 gm.	151 mm.	0.400 gm.	♀	—	0.025 gm.	—	Complete thymectomy. Infective lesions.
U3	186 dys.	14 gm.	111 gm.	—	0.318 gm.	♀	—	0.030 gm.	—	Complete thymectomy.
U4	68 dys.	14 gm.	36 gm.	124 mm.	0.220 gm.	0.180 gm.	0	0.020 gm.	—	Incomplete thymectomy. Accessory thymus. Infective lesions.
U5	186 dys.	15 gm.	116 gm.	—	0.669 gm.	♀	—	0.036 gm.	0.178 gm.	Control. Suppurative foci in lungs.
U6	185 dys.	14 gm.	152 gm.	—	0.472 gm.	1.124 gm.	Active	0.030 gm.	0.202 gm.	Control. Normal organs.
U7	68 dys.	15 gm.	38 gm.	129 mm.	0.166 gm.	0.296 gm.	0	0.017 gm.	0.070 gm.	Control. Probably infected.

The bones and teeth were reserved for chemical examination and were not studied histologically. There were no gross changes.

The foregoing protocols show that rats may live without thymus tissue and in apparent health for at least 186 days. The differences in weight between the thymectomized rats and the controls are in all likelihood referable to infections, histological evidence of which was found in rats U1 and U4. Moreover, a sex difference of twenty-five grams at this age is to be regarded as normal. The great retardation of growth in rats U4 and U7 emphasizes the need of caution in attributing such effects to the removal of the thymus, rather than to incidental disease.

The following experiment offers further evidence in favor of the negative results of thymus extirpation.

Litter O.[2]—This litter consisted of six rats, upon four of which thymectomy was performed on July 17, 1913. During the following weeks all the animals received prophylactic injections of a vaccine of *B. enteritidis*. With the exception of one rat, O4, which developed bloody diarrhea and died after four weeks, all the rats of this litter gained steadily in weight and remained healthy. Rats O1, O2, and one of the controls, O5, were killed on Nov. 15, 1913, 120 days after the operation. Rat O3 and the remaining control, O6, were killed eleven days later. In none of the operated rats were there found rests of thymic tissue or accessory glands. It is unnecessary to give the detailed protocols, since a careful microscopic examination of the bones and teeth showed no abnormal conditions. The calcification of the dentine of the incisors is shown for comparison with that in rachitic rats in figures 1a and 1b.

The following protocols serve as examples of the second group, in which both operated and control animals showed distinct alterations in the bones and teeth.

Litter X.—This litter consisted of six rats, four of which were operated on Oct. 24, 1913, the remaining two being kept as controls. The estimated age at the time of operation was sixteen days.

Rat X1.—On Nov. 12, seventeen days after thymectomy, a portion amounting to about one fourth of the entire thymus of rat X6 was removed and implanted into the peritoneal cavity of X1. No ill effects followed the implantation until Dec. 1, when the rat was found lying upon its side, suffering from general convulsions. The forefeet were strongly flexed, the hind legs extended and paretic. The rat was killed with ether.

Autopsy.—Well nourished male; weight 36 gm. All operative wounds completely healed. Abdomen prominent, intestines distended. No peritonitis. Careful search shows no trace of implanted thymic tissue. Spleen small. No other visceral changes.

[2] Tabulated weights and measurements and weight chart of this litter are given in the previous article (1).

Cultures from heart blood remained sterile.

Microscopic Examination.—Liver normal, no necroses. Spleen follicles small. Many megakaryocytes. Kidney and pancreas normal. Testicular tubules lined with indifferent epithelium, showing no mitoses. No meningitis. Ganglion cells of motor area show no changes recognizable when stained with hematoxylin and eosin. Vesicles of thyroid large and filled with abundant colloid. Complete series of neck organs; every tenth section mounted. Later, intermediate sections including all suspicious areas were also examined. No undoubted masses of thymic tissue were found. A small nodule situated between the esophagus, descending aorta, and right bronchus, at first interpreted as a thymus rest, was subsequently concluded to be a lymph node. It showed a fairly distinct peripheral lymph sinus, and complete absence of large epithelial cell complexes and formed Hassall bodies. In and about the hypertrophic cervical lymph nodes were many eosinophilic polynuclear leucocytes. No mitoses were found in the parathyroids.

The bones and teeth were fixed in Orth's fluid and decalcified in 5 per cent. nitric acid.

No rachitic changes were present in the femur or tibia. The epiphyseal lines were narrow and regular; the layer of proliferating cartilage cells at the lower epiphysis of the femur were only two to three cells deep. The trabeculæ of the spongiosa were far apart and narrow, the osteoblasts flat and inconspicuous. The osteoid zone was extremely narrow, in many places not recognizable. The lymphoid marrow extends to the epiphyseal cartilage in the femur. The lower portion of the tibia is occupied by fatty marrow. In the lymphoid marrow of the femur, there are scattered areas of necrosis, similar to those found in infections with *B. enteritidis*.

Sections of the upper and lower incisors show nothing abnormal. The calcification of the dentine is complete, save for the normal narrow calcium-free zone.

The interesting point in this protocol is the onset of convulsions and a tetanoid condition after removal of the thymus and subsequent isotransplantation into the peritoneal cavity. It does not seem probable that the convulsions were due to intoxication following the absorption of the thymus tissue. At the time of death, the implanted fragment had apparently been entirely resorbed. Further, the experiment has been repeated in other rats, and in no case were similar symptoms observed. The presence of focal necroses in the bone marrow suggests an infection as a more probable cause for the nervous symptoms.

The absence of bone lesions, in spite of complete extirpation and lack of accessory tissue, is to be noted.

Rat X2.—On Nov. 12 a small fragment of thymus from rat X6 was implanted beneath the skin of the abdominal wall. The small nodule disappeared after a few days, and no remains of it were found at autopsy. There were no

convulsive symptoms or other evidence of illness following the implantation. The rat remained well, but was behind the remaining animals of the litter in size and weight. It was allowed to live 151 days after thymectomy, and was then killed together with control rat X5.

Autopsy.—Poorly nourished, eyegrounds pale, slight exophthalmos. Internal organs normal. In the thymic region there were five or six lymph nodes and fat; no gross remains of thymus.

The ribs show fairly marked deformity. The zone of growing cartilage is broad and translucent, and there is distinct nodular swelling at the junction of cartilage and bone. There are also several opaque, fusiform, whitish swellings along the shafts of the ribs. The femur is 5 mm. shorter than that of X5, the control, and thicker, but not noticeably deformed. The teeth show no gross changes, aside from a notching of the cutting surface of the upper incisors.

Microscopic Examination.—Neck organs cut in complete series. A small thymus IV is found at the lower pole of the right thyroid lobe. It contains no formed Hassall bodies, but there are many epithelial cells with large pale nuclei in the medullary portion. The smooth margin and the absence of follicles and peripheral lymph sinus easily distinguish the tissue from lymph gland. It can be traced through only six sections. There is a large accessory parathyroid on the left side, lying above and external to the thyroid. All parathyroids show numerous mitoses, one or two to each immersion field.

The liver is free from necroses. The splenic follicles are of moderate size and composed chiefly of large lymphocytes. The pulp shows groups of erythroblasts and fairly numerous eosinophilic mononuclears and megakaryocytes. There is evidently active hematopoiesis. In the lung there is hyperplasia of the peribronchial lymphatic tissue. Adrenal medulla shows intense chromaffin staining of cells and capillary contents. It does not differ from that of the control.

In the ribs the zone of proliferating cartilage cells is about 0.4 mm. in width (normal width about 0.1 mm.). By actual measurement with the projection apparatus, it is found to be about six times as broad as that of a normal control. It is composed of fourteen to sixteen swollen cells. The margin is uneven and irregular columns and groups of cartilage cells in which the ground substance stains like osteoid are found amongst the trabeculæ of the spongiosa. These are wide, irregular in their arrangement, and composed in the neighborhood of the epiphysis almost wholly of a homogeneous osteoid. Only the central portions take a bluish stain and show typical bone structure. The marrow spaces are narrow, and the marrow is fibrous. The osteoblasts are very high and conspicuous. The cortex is broad, and the marrow cavity of the shaft correspondingly reduced. The nodular swellings seen in the gross correspond to localized thickenings of the compact bone. The epiphyseal cartilage of the femur is very convoluted, and there are masses of swollen cells projecting amongst the trabeculæ of the spongiosa. The width of the proliferating zone varies. It is from six to twelve cells deep. The spongy bone is dense, the marrow spaces reduced in width.

The incisor teeth show pronounced changes in the calcification of the dentine, both in the upper and lower incisors. The essential feature is a defective and irregular calcification, with ingrowth of blood vessels into the calcium-free dentine. On the concave side of the upper incisor, there are alternating striæ of

calcified and calcium-free dentine. No lesions are found in the enamel epithelium. The changes in the teeth are illustrated in figures 2a, 2b, 2c, and 2d.

Rat X5.—Control, male. Killed with ether at the same time as X2.

Autopsy.—Well nourished, apparently healthy rat. No gross lesions of internal organs, ribs, femora, or teeth. Testes small, undescended.

Microscopic Examination.—Liver normal, no necroses. Splenic follicles about the same size as those of X2, and of similar structure. Mitoses are not very numerous. Megakaryocytes and eosinophils abundant in pulp. Adrenals show intense staining of chromaffin elements and of capillary contents. Testicular tubules for the most part devoid of differentiated spermatic cells, being lined with indifferent syncytium showing no mitotic figures. A few tubules show differentiation up to the formation of spermatids. The interstitial cells are hyperplastic. Thyroid vesicles slightly smaller, epithelium higher, than in the thyroid of X2. Parathyroids show mitoses (two or three in each section), but less numerous than in X2.

Two ribs were examined. One shows much more pronounced changes than the other. The lesions in the more severely affected rib are identical with those described in the rib of X2. The width of the growing cartilage is increased, and the disarrangement of the cartilage cells is extreme. There is an excessive amount of spongy bone in the region of the epiphysis, the trabeculæ having a wide osteoid margin. The marrow is fibrous in character. The femur also shows similar changes.

The incisors show a marked defect in the calcification of the dentine, with ingrowth of cells and blood vessels from the odontoblast layer, as shown in figures 3a, 3b, 3c, and 3d. The enamel epithelium of the upper incisors is interrupted at one point and folded in upon itself. The enamel in this region has fallen out.

The comparative weights and measurements of rats X2 and X5 are given in table II.

TABLE II.

Rat.	Duration of experiment.	Weight at operation.	Weight at death.	Length.	Spleen. Weight.	Spleen. Length.	Adrenals. Absolute weight.	Adrenals. Relative weight.	Weight of testes.	Weight of thymus.	Remarks.
X2	151 dys.	19 gm.	86 gm.	150 mm.	0.333 gm.	27 mm.	0.022 gm.	$\frac{1}{3999}$ gm.	♀	—	Complete thymectomy. Small thymus IV.
X5	151 dys.	16 gm.	143 gm.	166 mm.	0.438 gm.	30 mm.	0.027 gm.	$\frac{1}{5300}$ gm.	0.374 gm.	0.143 gm.	Control. Cryptorchid.

There is a difference of weight of fifty-seven grams in favor of the control rat, and of sixteen millimeters in length. Both the operated rat and the control show marked lesions in the teeth and skeletal system.

The spontaneous occurrence of such lesions in the control rat

makes it highly improbable that they are in any way related to a loss of thymic function. Furthermore, if one is to ascribe the retardation in growth in rat X2 to the lack of thymus, one must assume that the presence of an accessory gland is not sufficient to ward off the effects. This would not coincide with Klose's assumption that such small lobules of accessory thymus tissue are capable of preventing the deprivation symptoms, and that the negative results of extirpation can be explained in this way.

The remaining rats of the litter, X3 and X4, and the control, X6, were killed 156 days after the operation.

Rat X3.—Large, well nourished male. Nothing abnormal noted in viscera, bones, or teeth. Many large lymph nodes in substernal region, but no gross remains of thymus.

Microscopic Examination.—Liver, spleen, adrenals, lungs, and kidneys normal. Testes show active spermatogenesis. Interstitial cells large, distinct, and numerous.

Neck organs cut in complete series. A lobule of thymus tissue is found lying along the trachea 1 cm. above the bifurcation. It is elongated and narrow, measuring approximately 1.5 by 0.3 by 0.075 mm. The thymus structure is distinct, although there are few fully formed Hassall bodies. At one pole are several vesicles lined with cuboidal epithelium, and containing basic staining colloid material. The right parathyroid lies external to the thyroid, and is irregular in shape, partially encircling the carotid. Mitoses are not infrequent.

In the upper incisors the calcification of the dentine is practically complete on the convex surface throughout the whole length. Near the base of the tooth, the border of the calcified portion is slightly wavy, and there are a few ingrowing blood vessels. The concave edge also shows good calcification of the dentine, although in the middle portion there are a few calcium-free streaks, and in places the calcium is deposited in globular form. The odontoblast layer is well formed through the entire extent of the pulp cavity on both sides. The enamel is wanting in the sections, but the emaloblasts are normal.

Normal chondrocostal junctions in several ribs examined. There are four to eight growing cartilage cells, regularly disposed. The osteoblasts are well formed. There is no excess of osteoid tissue.

Rat X4.—Thymectomy.

Autopsy.—Fairly well nourished female. No changes in internal viscera. No macroscopic remains of thymus. Definite lesions are seen in the ribs, similar to those described in X2. The chondrocostal junctions are swollen, and there is sharp angulation of the lower ribs. The zone of growing cartilage is broad, and the adjacent part of the shaft for a distance of about 2 mm. is greyish white and soft. Four of the ribs show on the pleural surface, about 1 cm. from the junction with the cartilage, pearly, cartilaginous swellings. Teeth and femora show no gross changes.

Microscopic Examination.—Liver, kidneys, and adrenals normal. Splenic follicles slightly smaller than those of the control, X6. Few mitoses. Many

eosinophils and much pigment in pulp. Lungs show many eosinophils in edematous tissue about larger blood vessels.

The upper incisors show changes similar to those described in rats X2 and X5, but somewhat less marked. The defective calcification of the dentine, on the convex surface, near the base of the tooth, is marked, about one third of the entire width being entirely calcium-free. In the middle portion, about two fifths of the dentine is uncalcified. Here, and also towards the tip, the calcium is deposited in laminæ. There is an abundant ingrowth of capillaries from the odontoblast layer. On the concave side, near the base, half the dentine is calcium-free. Towards the middle and distal portion, the calcification is still more incomplete, and in the form of alternating calcium-free and calcium-containing layers. The lower incisors show similar changes, especially marked on the concave surface.

The growing cartilage of the ribs presents an even convex surface towards the shaft. The rows of cartilage cells are six to ten cells deep. The spongiosa is composed of very broad trabeculæ with wide osteoid zones, and high osteoblasts. The pearly swellings observed in the gross correspond to localized thickenings of the compact bone.

The zone of growing cartilage of the femur at the lower epiphysis is convoluted and slightly increased in breadth, but not markedly irregular. The spongiosa is formed of coarse trabeculæ with a relatively broad osteoid zone and distinct osteoblasts.

Neck organs, cut in complete series, show no thymus tissue, with the exception of a minute accessory lobule below the inferior pole of the right parathyroid gland and lying within the capsule of the thyroid. This is composed of large cells with very pale nuclei, between which are scattered small cells of the lymphoid type. The lobule contains also a few eosinophils, and abundant brownish pigment. There are no Hassall bodies or large epithelial cell complexes. The tissue can be traced through only five sections. There are two parathyroids, both within the thyroid. The epithelial cells show fairly numerous mitoses, two or three to each section. The thyroid vesicles are small, save at the surface of the gland; the cells are columnar, but not infolded. The colloid is abundant, but vacuolated.

Rat X6.—Control.

Autopsy.—Large, well nourished rat, showing no changes in the internal organs. There is slight beading of the ribs, with a broad greyish zone adjacent to the cartilage. The femora and teeth appear normal.

Microscopic Examination.—Liver, kidneys, and adrenals normal. The splenic follicles are slightly larger than those of rats X3 and X4. Mitoses are not numerous. In the pulp are many erythroblasts and eosinophils. Some of the testicular tubules show active spermatogenesis with formed spermatozoa in the lumina. Most of the tubules, however, are lined with indifferent epithelium showing no mitoses. Interstitial cells are somewhat more abundant than in the testis of rat X4.

In the upper incisors the odontoblasts are well formed, extending through the entire length of the pulp. Calcification of the dentine is not abnormal, save that the odontoblast layer is rather wavy. The uncalcified predentine is narrow.

Ribs and femur show nothing abnormal.

The comparative weights and measurements of rats X3, X4, and X6 are summarized in table III.

TABLE III.

Rat.	Duration of experiment.	Weight at operation	Weight at death.	Length.	Spleen.		Adrenals.		Weight of testes.	Remarks.
					Weight.	Length.	Absolute weight.	Relative weight.		
X3	156 dys.	19 gm.	195 gm.	199 mm.	0.447 gm.	31 mm.	0.027 gm.	$\frac{1}{7240}$	1.382 gm.	Incomplete thymectomy.
X4	156 dys.	16 gm.	110 gm.	165 mm.	0.311 gm.	31 mm.	0.028 gm.	$\frac{1}{3917}$	♀	Complete thymectomy. Minute thymus IV.
X6	156 dys.	20 gm.	180 gm.	205 mm.	0.542 gm.	34 mm.	0.021 gm.	$\frac{1}{8571}$	0.440 gm.	Control. Hypoplasia of testicles.

Rat X4 offers a further illustration of the fact that rats may survive complete extirpation for many months without becoming cachectic. It seems improbable that the minute accessory gland, less than fifty microns in diameter, could take over the functions of the entire thymus without showing evidence of active hyperplasia.

The rachitic lesions in this rat were marked, and as will be evident from subsequent protocols, this disease in rats is frequently, though not always, accompanied by a retardation of growth. There is, therefore, little ground for attributing the diminution in size and weight, as compared with the control, to the lack of thymus. The normal sex difference must also be taken into account at this age.

Litter Y.—This litter, consisting originally of eleven rats, was born in the laboratory of stock animals, all the rats previously studied having been obtained from Granby, Mass. The rats were undersized, and stood the operation poorly, only three thymectomized and three controls surviving.

Rat Y2.—Thymectomized Oct. 27, 1913. Weight 10 gm. Gained slowly and remained undersized. Killed on Mar. 13, 1914, 137 days after extirpation of the thymus.

Autopsy.—Internal organs normal save for suppurative foci in the lower lobe of the right lung. Testes large and descended. No alterations of the bones and teeth were recorded.

Microscopic Examination.—Lungs show bronchiectases and bronchopneumonia. Active spermatogenesis. Interstitial cells not numerous. Liver, adrenals, and kidneys normal. Spleen shows active blood formation.

Extirpation of thymus probably complete. The only suspicious tissue lies in a lobule of fat near the hilus of the left lung, where there is a circumscribed collection of mononuclear cells with a few larger elements, which can be traced

through sixteen sections. There are no epithelial cell complexes or Hassall bodies, but the tissue does not have a peripheral lymph sinus or follicles. It is probably a newly forming lymph node. The thyroid is composed of large vesicles with abundant colloid and flat epithelium. In the fat tissue external to it, are a few closed ducts lined with high cylindrical epithelium (postbranchial ducts).

The line of growing cartilage of the ribs is broad, with a convex, irregular surface directed towards the shaft. It is composed of eighteen to twenty cells in rows, with masses of cartilage cells projecting amongst the trabeculæ of the spongiosa. These are numerous, broad, with a wide osteoid margin, and high conspicuous osteoblasts. The marrow spaces in the region of the cartilage are narrow, the vessels hyperemic and surrounded by spindle cells.

Upper epiphysis of tibia. No definite rachitic changes. The zone of growing cartilage is slightly narrower than that of the control. The cells are regular in their arrangement, as are the trabeculæ. Bone formation is very active. The osteoid zone about the trabeculæ is slightly increased in width. This is especially clear in the epiphyseal center of ossification.

The epiphyseal line of the femur is regular on the whole, but there are a few projections of cartilage amongst the trabeculæ of the spongiosa. The osteoid is increased in amount.

The calcification of the dentine of both upper and lower incisors is somewhat defective, but there is no vascularization of the calcium-free dentine. The enamel epithelium is not altered.

Much more striking were the skeletal and dental changes in rat Y8, the protocol of which follows.

Rat Y8.—Thymectomy on Nov. 1, 1913. Weight at operation 14.6 gm. Following the operation, and until the rat was killed on Mar. 2, 126 days after extirpation of the thymus, it lagged far behind the remaining rats of the litter in weight and size. The hair was rough and sparse; the head seemed relatively large as compared with the body. On Jan. 17, it was noted that the upper incisors were very long and thick as compared with the lower, which were small and delicate. On Feb. 28 the right upper incisor was broken off just beyond its emergence from the gum.

Autopsy.—Poorly nourished, stunted male. Weight 42 gm. The internal viscera were normal, with the exception of the right lung, which was atelectatic in its upper portion. No gross remains of thymus. The thorax is greatly deformed by the presence of a deep groove along the line of the chondrocostal junctions. From the pleural surface there are seen, especially on the lower ribs, nodular protuberances at corresponding points. There is thus a pronounced rachitic rosary. The bones of the lower extremities are softer and more pliable than normal, and there is distinct swelling about the epiphyses of the knee-joints.

Cultures from the heart blood and spleen on slant agar remained sterile.

Microscopical Examination.—Liver free from necroses. Splenic follicles large and sharply circumscribed. Many of the lymphoid cells are of the large mononuclear type. Mitoses are scarce, and there are no distinct germinal centers. In the pulp there are many islands of normoblasts and groups of myelocytic eosinophils. Adrenals show the usual intense chromaffin staining. Testicular tubules are lined with four or five rows of cells, most of them spermatoblasts

in active division. There are a few spermatids, but no ripe spermatozoa. The appearance is that of a testis just before the onset of spermatogenesis. Some of the bronchi contain dense collections of eosinophils. The pulmonary arteries are thickened, their lumina contracted, the muscular coat appears hypertrophied. Some of the vessels are practically obliterated.

Complete series of neck organs. There is no tissue which can be identified as thymus. There are no accessory thymus lobules. The parathyroids are large; no mitoses are found. Thyroid shows usual structure with abundant colloid.

The upper incisors were sectioned through the fractured stump. The pulp is hyperemic and largely necrotic. The calcification of the dentine is both irregular and incomplete. The line of junction between odontoblasts and predentine is sinuous, and in many places the odontoblast cells penetrate the uncalcified tissue. The calcified portion of the dentine is laminated, the calcium being in the form of coarse globules. In another section there are alternating zones of calcified and calcium-free dentine. Isolated cells and occasionally capillary loops reach the outer, imperfectly calcified zone. Sections through the unfractured upper incisor show also defective calcification. Near the root, there is the same stratification as in the fractured tooth. In the middle portion, just after it emerges from the socket, the section passes outside the pulp canal. In this region there is a large area of calcium-free dentine. Near the convex surface, within the calcified strata, there are a number of globular yellowish masses. In sections stained with thionin-picric acid, it is seen that the dentine filaments are interrupted at the site of these globular deposits. Near the root, the congested pulp vessels penetrate the uncalcified dentine as papillary ingrowths. About one half of the dentine on the convex surface of the lower incisors is free from calcium. The odontoblasts are poorly formed and irregular. There is no marked ingrowth of blood vessels. On the concave side the calcification is still more imperfect.

The zone of growing cartilage of the humerus at the lower epiphysis is widened, and irregularly prolonged amongst the trabeculæ. These are very broad and the marrow spaces correspondingly narrow. The trabeculæ are composed in the neighborhood of the epiphysis of a narrow, ossified central portion, distinctly laminated, with angular bone corpuscles, surrounded by a very broad, homogeneous, osteoid zone in which the nuclei of the bone corpuscles are larger and more rounded, and quite irregularly distributed. The osteoblasts are conspicuous and well formed. There is no increased osteoclastic resorption. The changes at the lower epiphysis of the femur are similar in character, but more pronounced than in the humerus. Numerous sections from four different ribs show similar changes. There is marked deformity at the junction of cartilage and bone, where the rib measures 3 mm. in diameter. The junction of the cartilage with the shaft is in most places sharply marked off. There is a large central mass of hypertrophic cartilage, in some places twenty-five cells deep, and two shorter lateral spurs. Near the pleural surface, smaller irregular groups of cartilage cells are surrounded by osteoid-staining matrix. The adjacent cancellous bone is composed of stout trabeculæ formed entirely of pink staining osteoid, separated by very narrow clefts and spaces. They are ranged, in the region where the rib is sharply angulated, at right angles to the cartilage columns.

The distribution of the bone corpuscles through this mass of osteoid is haphazard. Farther along the shaft, the trabeculæ of the spongy bone follow the long axis of the rib. They are all greatly thickened, as is the corticalis, and well calcified only in their central portions. In thionin-picric acid-phosphotungstic acid-stained preparations, the broad osteoid, blue staining zone appears with great distinctness. The marrow cavity is much reduced in width. Near the epiphysis the marrow is fibrous; further along, it has the normal lymphoid appearance.

Two control rats of the litter, Y7 and Y9, showed alterations in the teeth and bones comparable with those observed in the thymectomized rats, though somewhat less pronounced. A third control, Y10, which had complete visceral transposition, was not histologically examined.

Rat Y7.—Control. Killed Apr. 7, 1914. Poorly nourished female, weighing 115 gm. Internal organs show no lesions, with the exception of the lung. The right middle lobe is converted into a mass of cheesy abscesses, separated by scar tissue. The ribs show slight beading at the chondrocostal junctions, with angular deformity in the region of the diaphragmatic attachment. The zone of growing cartilage is broad. No gross changes in femora or teeth.

Microscopic Examination.—Internal viscera, aside from lung lesions, show nothing abnormal.

In the upper incisors calcification of the dentine is imperfect, both on the convex and on the concave surfaces. The odontoblast layer is broad, but ill defined, and on the concave side cells are seen to penetrate the calcium-free dentine for varying distances. Both surfaces of the lower incisors show alternating layers of calcium-containing and calcium-free dentine. The calcium is deposited in the form of coarse globules.

TABLE IV.

Rat.	Duration of experiment.	Weight at operation.	Weight at death.	Length.	Spleen. Length.	Spleen. Weight.	Adrenals. Absolute weight.	Adrenals. Relative weight.	Weight of testes.	Remarks.
Y2	150 dys.	10 gm.	85 gm.	145 mm.	26 mm.	0.220 gm.	0.013 gm.	$\frac{1}{6538}$	1.483 gm.	Complete thymectomy.
Y8	121 dys.	14.5 gm.	42 gm.	127 mm.	25 mm.	0.312 gm.	0.020 gm.	$\frac{1}{2100}$	0.332 gm.	Complete thymectomy.
Y9	150 dys.	16 gm.	118 gm.	170 mm.	32 mm.	0.380 gm.	0.030 gm.	$\frac{1}{3933}$	1.980 gm.	Control. Abscesses of lungs.
Y7	168 dys.	16 gm.	115 gm.	157 mm.	34 mm.	0.333 gm.	0.043 gm.	$\frac{1}{2674}$	♀	Control.

The growing cartilage of the femur is narrow, being composed of only two to four rows of cells in the lower epiphysis. Both in the compact and in the spongy bone, the osteoid margin is very broad and distinct, especially about the

perforating vessels. The ribs show no deformity in the sections examined, but there is a distinct increase in the width of the osteoid zone about the trabeculæ.

Rat Y9.—Control, male. Killed Mar. 13, 1914. Weight 118 gm. There were no lesions of the internal viscera. The alterations of the teeth were insignificant (slight lamination of the calcium on the convex surface of the lower incisors), but there was distinct irregularity and thickening of the cartilage at the lower epiphysis of the femur and at the upper epiphysis of the tibia. The single rib examined was practically normal.

The comparative weights and measurements of litter Y are given in table IV.

To the foregoing protocols of thymectomized and control rats may be added the brief description of two unoperated rats taken from the stock cages, which showed lesions in the bones and teeth identical with those described above.

The first of these (rat 50) was greatly stunted; there was a marked disproportion between the size of the head and that of the body. The approximate weight was 50 gm. The hair was rough and sparse. The teeth were somewhat chalky, but not greatly altered. There was an extreme deformity of the thorax (figure 5), resembling that noted in rat Y2. There was also marked kyphosis and lateral curvature. There were many whitish nodules along the shafts of the ribs, especially in the paravertebral lines. The ribs were very soft, and on cross-section the marrow cavity seemed almost obliterated owing to the great thickening of the cortex of the bone. At the chondrocostal junctions were nodular projections several millimeters in thickness. The femora were short, and very broad in the region of the epiphyses. The upper third of the tibiæ was bowed anteriorly.

Microscopic Examination.—The superficial layer of dentine of the upper incisors is well calcified; in the middle zone, the calcium is in the form of globular masses of large size, and the inner zone, comprising about one third of the total thickness of the dentine, is calcium-free. Towards the apex the calcification is more complete. The lower incisors exhibit more striking changes, especially on the concave surface where about half the dentine is free from calcium. The vessels of the pulp are extremely congested, and the odontoblast layer is broken up in many places by fresh capillary hemorrhages. Towards the apex of the tooth, the layer of odontoblasts becomes entirely disorganized, with the formation of numerous ragged cavities, possibly the effect of gas formed during decalcification. The enamel epithelium is intact save in one place, where the cells are destroyed by hemorrhage.

The zone of proliferating cartilage of the ribs is composed of irregular masses of cells which fail to show the normal alignment into columns, and which extend a variable distance into the adjacent trabeculæ. The neighboring part of the rib is composed of a knob of anastomosing columns separated by very narrow Haversian spaces. The distinction between osteoid and calcified bone is not sharply brought out in the sections, but the rounded form of the bone corpuscles and their highly irregular distribution suggest deficient calcification. The osteoblasts form a continuous flat row covering the trabeculæ. There is no increased

lacunar resorption. Beyond this knob of tissue the bone is sharply bent upon itself. A tongue of cartilage grows into this angle from the external surface of the periosteum. The cortex of the rib is greatly thickened and formed of parallel trabeculæ which encroach upon the medullary cavity, which is correspondingly narrowed. The marrow is normal, save in the region of the junction where it is fibrous (figure 6).

In order to determine the possible infectious character of this disease, four half grown and apparently healthy rats were inoculated with a suspension of bone marrow from rat Y8. One of the inoculated rats, killed twenty-eight days after subcutaneous injection, showed striking alterations in the bones and teeth. There was in this rat no gross deformity of the thorax, but an evident widening of the translucent zone of proliferating cartilage, and the adjacent bony part of the ribs was opaque and slightly thickened. The femora were broad and thick, and so soft that they could easily be cut with scissors. The tibiæ were bowed anteriorly.

Changes in the teeth were first noted four days before the animal was killed. The basal portions of the upper incisors were found to be distinctly swollen, translucent, greyish, and rounded, sharply marked off from the distal third which was opaque and chalky white. During the next few days, the swelling and translucency extended almost to the tip of the tooth. The lower incisors were also less opaque than normally.

Microscopic Examination.—No changes were found in any of the internal viscera. The marked defect and irregularity of the dentine is shown in figures 4a, 4b, 4c, and 4d. It is seen that on the convex surface of the upper incisors the irregularity in the arrangement of the odontoblasts is such that these have apparently given rise to predentine both on their external and internal surfaces. They are thus disposed in a row on both sides of which is uncalcified dentine, and are connected by numerous cell strands with the pulp. The concave side of the tooth is calcium-free throughout its entire extent, and is pierced in many places by isolated spindle cells and occasionally by capillaries. The changes in the ribs and femora consisted merely of an increase in the width of the osteoid margins. No definite alterations were found in the cartilages.

DISCUSSION.

The foregoing protocols show that lesions of the bones and teeth were observed in thymectomized rats, in controls of the same litters, in stock animals, and in a rat inoculated with bone marrow from a thymectomized rat with lesions of the skeletal system.

There is little question that these lesions resemble closely those described by Morpurgo (5), in 1900, as infective osteomalacia of white rats. In his subsequent investigations, extending over many years, Morpurgo showed that in young animals, the spontaneous disease took the form of rachitis.

From the spinal cord of a spontaneously diseased osteomalacic rat, and later, from the spleen, liver, kidneys, and bones, Morpurgo isolated a Gram-positive diplococcus with a tendency to form chains in fluid cultures. With pure cultures of this microörganism, Morpurgo was able to produce rachitic lesions in young rats, and osteomalacia in adult rats in which epiphyseal growth had been completed.

The clinical course of the disease varied in different rats, in part owing to variations in the virulence of the cultures, in part apparently to variations in the susceptibility of individual animals. In some cases the rats became greatly emaciated, and died from the disease. Other animals lived for over a year, in which case the bone lesions showed evidence of healing.

The deformities produced also varied widely in different rats. They were found especially in the vertebral column, the thorax, the scapulæ, the posterior extremities, and the pelvis,—more rarely in the anterior extremities and the maxillary bones. The vertebral column was lordotic and scoliotic in the dorsal region, kyphotic in the sacral and cervical portions. The thorax was much depressed along the insertion of the pectoral muscles, and along the line of depression the ribs were enlarged in consequence of callus formation following infractions or complete fractures. Small beads of callus were also seen along the paravertebral line, or scattered irregularly along the course of the ribs. The cartilages were also tortuous, nodose, and yellowish.

The femora were rarely curved or bent, and only when the presence of callus indicated a previous fracture. The diaphysis was often greatly enlarged, the medullary cavity eccentric or narrowed. The tibia was often bent at an angle between the upper and middle thirds.

The microscopic examination of the bones disclosed a picture which was identical with that seen in human rickets or osteomalacia. Schmorl and others have accepted the morphological identity of the lesions in the rat with those of human rachitis.

I have not found in Morpurgo's numerous papers any reference to changes in the teeth. Weichselbaum (6), however, reported finding lesions in the teeth in three spontaneously rachitic rats, which he describes as follows:

"The dentine of the incisors nowhere shows a uniformly calcified zone, but the zone adjacent to the odontoblasts, *i. e.*, the younger layer, was completely bereft of calcium, and thus appeared pale red in hematoxylin-eosin preparations, whereas the older layers were either wholly calcium-free or unevenly calcified, in which case they contained numerous blue dentine globules separated by pale, so called interglobular spaces. One sees further that from the odontoblast layer, capillary loops penetrate more or less deeply into the dentine. In one of the two rats one of the upper teeth was broken off."

Weichselbaum calls attention to the close resemblance of these changes found in the spontaneous rachitis of rats, to the lesions found by Fleischmann (7) in the teeth of human rachitic subjects, and especially to the dental changes described by Erdheim (8) and Toyofuku (9) as following parathyroidectomy in rats.[3] Hohlbaum (10), also, confirming Erdheim's findings, noted in two spontaneously affected rachitic rats identical lesions in the teeth. Furthermore, Iselin (11), in his study of the effects of parathyroid extirpation, found rachitic-like changes in the epiphyses of those rats which survived for a longer period, and attributed them to the loss of parathyroid function.

Defective calcification of the dentine, and penetration of the uncalcified tissue by blood vessels and isolated cells from the odontoblast layer, regarded by Erdheim and his successors as characteristic of parathyroid deprivation, were observed also in many of the rats described above. In one rat, there occurred a spontaneous fracture of the tooth. Less marked in my rats was the infolding and disarrangement of the emaloblast layer; but this appears to be of secondary importance also in the lesions attributed to parathyroidectomy, and when present is probably due to mechanical causes.

The question arises, therefore, whether Erdheim, who was familiar with the spontaneous rachitic lesions of rats, was justified in attributing these changes to parathyroid insufficiency. Erdheim (12) has brought forward his transplantation experiments as crucial proof of the part of the parathyroids in normal calcification. The "*Transplantations-streife*" is believed to correspond exactly in its localization with the period of functional activity of the implanted parathyroid tissue.

This would be entirely convincing were it not for the fact that such alternating layers of calcium-containing and calcium-free den-

[3] This resemblance was accepted by Erdheim, who studied Weichselbaum's specimens.

tine not infrequently occur as spontaneous rachitic lesions. They are associated, no doubt, with remissions and exacerbations of the disease, such as have been described by Morpurgo. Figure 3 illustrates the striate deposition of the calcium in the incisor of a spontaneously rachitic rat.[4]

Without wishing to discredit the work of Erdheim, supported as it is by other facts indicating an influence of the parathyroids upon calcium metabolism, one cannot but emphasize the identity of these lesions attributed to the loss of parathyroid function, with those occurring in the spontaneous rickets of rats.

As regards the infectivity of the disease and the nature of the inciting organism, studies made in coöperation with Dr. J. G. Hopkins have not as yet led to conclusive results. The occurrence of rachitic lesions in a rat, examined twenty-eight days after inoculation of bone marrow from a spontaneously rachitic animal, is of doubtful significance, since many of the stock animals killed at this time were found to be rachitic. The recent experiments of Koch (13), in which typical rachitic lesions were produced in puppies by the intravenous injection of streptococci, indicate that the infective factor has to be reckoned with in experiments dealing with the effect of extirpation of ductless glands upon bone development in dogs as well as rats.

Sufficient evidence, we believe, has been brought forward to show that the bone changes described by Klose are in no way related to the loss of the thymus gland, but occur as the result of spontaneous disease. It is difficult indeed to understand why Klose should believe that his "experiments on rats acquire fundamental significance in view of the experiments of Morpurgo";[5] and why, in the same article, he should imply that rats are amongst those species which are practically immune to spontaneous rickets.[6]

As regards alterations in the weights and microscopical structure of the adrenals, testes, and spleen, which might point to a correlation between the thymus and these organs, nothing has been found to

[4] This appearance is pictured in figure 4, plate 6/7, of Toyofuku's paper (9), where it occurred in a rat, forty-six days after parathyroidectomy, in which no transplantation had been performed.

[5] Klose, *loc. cit.*, p. 4.

[6] Klose, *loc. cit.*, p. 7.

modify the negative conclusions reached in the previous paper. An analysis of the additional figures given therefore seems unnecessary.

CONCLUSIONS.

Rachitic changes in the bones and teeth occur in young albino rats as the result of spontaneous disease, possibly of infective origin. This disease is often, but not always, accompanied by an arrest of growth and malnutrition.

There is no valid reason for attributing such lesions to the loss of thymic function. In some of the rats showing the disease, an unusual number of mitoses were found in the parathyroid glands, but no evidence of injury to these structures.

It has not been found possible to confirm the statements of Klose and Magnini as to the fatal effects of complete thymus extirpation in rats.

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EXPLANATION OF PLATES.

Plate 22.

Fig. 1a. Rat O1. Thymectomy. Killed after 120 days. Upper incisor, convex side. D1 = calcium-free dentine. D2 = calcified dentine. O = odontoblasts.

Fig. 1b. Same tooth, concave side. Normal relations between calcified and uncalcified dentine.

Fig. 2a. Rat X2. Thymectomy; accessory thymus. Killed after 151 days. Upper incisor, convex side, about middle of tooth. Defective calcification of dentine, ingrowth of blood vessels from odontoblast layer. D3 = incompletely calcified dentine. C = capillary.

Fig. 2b. Concave side of same tooth. Same designations.

Fig. 2c. Lower incisor, concave side.

Fig. 2d. Lower incisor, convex side.

Plate 23.

Fig. 3a. Rat X5, control. Killed at same time as X2. Upper incisor, convex side.

Fig. 3b. Upper incisor, concave side. Deposition of calcium in layers.

Fig. 3c. Lower incisor, convex side.

Fig. 3d. Lower incisor, concave side. Alternating striæ of calcium-containing and calcium-free dentine.

Fig. 4a. Rat Y8(3). Injected subcutaneously with suspension of bone marrow of rachitic rat Y8. Killed after 28 days. Upper incisor. Incomplete calcification of dentine. Irregular disposition of odontoblasts. I = islands of uncalcified dentine. P = pulp.

Fig. 4b. Upper incisor, concave side. Dentine wholly calcium-free. Odontoblast layer indistinct.

FIG. 4c. Lower incisor, convex side.

FIG. 4d. Lower incisor, concave side. Dentine entirely uncalcified and convoluted. Ingrowth of odontoblasts and capillaries.

PLATE 24.

FIG. 5. Rat 50. Spontaneous rachitis. View of right half of thorax and vertebral column.

PLATE 25.

FIG. 6. Rat 50. Section of rib, low power drawing. K = zone of proliferating cartilage. K1 and K2 = islands of cartilage. O1 = osteoid tissue. O2 = calcified bone. P = ingrowth of periosteum. V = thickening of periosteum (old callus?). M = medullary cavity.

THE CALCIUM CONTENT IN BONES AND TEETH FROM NORMAL AND THYMECTOMIZED ALBINO RATS.*

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(From the Biochemical Laboratory of Columbia University, at the College of Physicians and Surgeons, New York.)

This paper deals with the proportions of calcium in the bones and teeth from rats used by Dr. Pappenheimer in the experiments described in the preceding paper.[1] After the conclusion of the autopsy by Dr. Pappenheimer, and the removal from the rat of any parts required by him, we took all the teeth and also the femurs and tibias from each animal. The teeth individually were carefully pulled out of place after the grip of the bones and membranes that held them had been broken by suitable paring and excision. Each tooth was cleaned with filter paper. Adherent muscle was cut from the leg bones, which were then gently scraped clean with a scalpel.

After free exposure to the air, for prompt desiccation at room temperature, the teeth and bones were separately crushed and pulverized in a deep porcelain mortar. When, for any reason, we were unable to obtain all the teeth with both the femurs and the tibias from an animal, we used these materials from one side only, and proceeded on a 50 per cent. basis, in this way preventing any analytic irregularities that might have resulted from the use of masses that were not anatomically equivalent in kind and proportion.

After the pulverized material had been uniformly mixed and dried to constant weight at 100° to 110° C., an accurately weighed portion of it, practically all the material in each case, was decomposed in a large Kjeldahl digestion flask of Jena glass, with a mixture of equal parts of concentrated sulphuric and nitric acids (Neumann's method).[2] The clear, colorless, concentrated, acid solution, freed from most of the mechanical excess of residual sulphuric acid, was then diluted to 500 cubic centimeters or one liter, and kept in tightly stoppered bottles. Calcium was determined in duplicate by

* Received for publication, August 10, 1914.

[1] Pappenheimer, A. M., *Jour. Exper. Med.*, 1914, xx, 477.

[2] Thierfelder, H., in Hoppe-Seyler, F., Handbuch der chemischen Analyse, 8th edition, Berlin, 1909, 539.

McCrudden's method[3] in 100 cubic centimeter portions of each solution prepared in this way.

We are indebted to Dr. Pappenheimer for the accompanying tabular statement of his data (table I), in which he has inserted our percentage results for calcium in the water-free bones and teeth.[4]

TABLE I.

Data Pertaining to Albino Rats Described in the Preceding Paper.[5]

Rat.	Thymectomy.	Duration of experiment, in dys.	Weight at death, in gm.	Rachitic lesions.		Percentage of calcium.[6]		Remarks.
				Bones.	Teeth.	Bones.	Teeth.	
U_1	Complete	185	97.3	None	None	22.61	27.48	No histological examination of bones or teeth.
U_3	Complete	186	111.0	None	None	24.01	28.97	No histological examination of bones or teeth.
U_5	Control	186	116.0	None	None	23.16	27.93	No histological examination of bones or teeth.
U_6	Control	185	152.0	None	None	22.74	28.29	
X_3	Incomplete	156	195.5	None	Slight	21.37	26.51	
X_4	Complete	156	110.5	Moderate	Slight	20.89	28.61	Minute thymus IV.
X_5	Control	151	143.0	Marked	Marked	18.92	—	
X_6	Control	156	180.0	None	Slight	22.84	28.12	
Y_2	Complete	150	85.0	Marked	Moderate	17.85	28.53	
Y_7	Control	168	115.0	Moderate	Moderate	23.14	27.09	
Y_9	Control	150	118.5	Moderate	Moderate	18.68	27.23	
W_2	Complete (?)	192	240.0	None	None	24.40	28.31	Series of neck organs incomplete.
W_4	Control	192	240.0	None	None	23.75	28.99	
Z_1	Incomplete	166	145.0	None	Very slight	22.29	28.43	
Z_4	Control	166	123.0	None	None	23.13	27.84	
50	Stock	—	—	Marked	Slight	23.41	26.44	Inoculated with bone marrow from spontaneously rachitic rat.
53	Stock	—	90.0	Moderate	Marked	18.15	—	Spontaneous rachitis.
55	Stock	—	75.0	Moderate	Marked	20.66	25.49	Inoculated with bone marrow from spontaneously rachitic rat.

[3] McCrudden, F. H., *Jour. Biol. Chem.*, 1911, x, 187. Neuberg, C., Der Harn, sowie die übrigen Ausscheidungen und Körperflüssigkeiten, Berlin, 1911, i, 168.

[4] We did not remove the lipins, on the assumption that variations in their proportions could not be great enough materially to affect the data obtained on a water-free basis.

[5] Pappenheimer, A. M., *loc. cit.*

[6] Calcium content is expressed as calcium (Ca), not as lime (CaO).

Data pertaining to average percentage amounts of calcium in the water-free bones and teeth, classified for various groups of the rats, are given in table II.

TABLE II.

Data Pertaining to the Content of Calcium, in the Bones and Teeth, for Various Groups of the Rats Referred to in Table I.

Groups of rats.	Bones.	Teeth.
	Percentage of calcium.	
1. Non-rachitic and slightly rachitic	23.03	27.99
2. Moderately rachitic and markedly rachitic	20.21	27.08
3. Completely thymectomized (excluding W_2)[7]	21.34	28.40
4. Control and incompletely thymectomized[7]	22.00	27.83
5. Rachitic (stock)	20.74	25.96

The foregoing chemical data agree, in the main, with the corresponding histological findings reported in Dr. Pappenheimer's paper. Our results indicate that the bones and teeth of the rats which were found by Dr. Pappenheimer to be moderately rachitic and markedly rachitic, contained in general less calcium than those from the non-rachitic and slightly rachitic animals. The lack of any particular trend of calcium content in the bones and teeth from the thymectomized rats, as shown by both the individual results in table I and the group averages in table II, makes it impossible to ascribe to thymectomy, in these animals, any special influence upon the processes of calcification in the bones and teeth subjected to analysis. The validity of this negative conclusion is emphasized by the low values for calcium content in the bones and teeth from the unoperated rachitic (stock) animals. That such spontaneously rachitic rats happened to be among the number selected for Dr. Pappenheimer's experiments, and that rachitic lesions observed in the thymectomized animals were due to causes independent of thymectomy, are necessary inferences under the circumstances. It is possible, of course, that determinations of phosphate content might have afforded indications of a special influence of thymectomy.

[7] Rat W_2 (table I) evidently belongs to group 4 in this table. If the figures for the calcium content pertaining to rat W_2 are added to those of group 4 in this table, the average values for calcium become 22.22 and 27.87 per cent., respectively.

It is assumed by Dr. Pappenheimer and by us that the mixed food of the rats, which was given in abundance and eaten freely, was ample in quantity, in quality, and in metabolic availability for the maintenance of the processes of normal calcification in each animal. One cannot be certain of such an outcome in experiments of this kind, however, unless the animals are individually maintained under suitably controlled metabolic conditions on diets of definite composition, of known quantity, and of proved nutritional adequacy. We require also, for correct interpretations of analytic data on calcification, more exact chemical knowledge of the variations in the composition of the bones and teeth of normal albino rats of different ages under given metabolic conditions. It is our intention to investigate in this laboratory, as a part of a plan of research in progress, the facts in these particular connections, with special reference to the possible establishment of definite physiological standards on which further experimental work with albino rats may be conducted more accurately.

The analyses reported in this paper were conducted during the progress of experiments by us on the influence of thyroparathyroidectomy on dentition in albino rats, under the auspices of the Dental Society of the State of New York. In the execution of the analyses pertaining to that work, and also of those reported here, we have enjoyed the assistance of our associates, Dr. Alfred P. Lothrop and Mr. William A. Perlzweig.

THE INFLUENCE OF VARIOUS SUBSTANCES ON THE GROWTH OF MOUSE CARCINOMA.*[1]

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(From the Department of Pathology of the Barnard Free Skin and Cancer Hospital, St. Louis.)

We carried out the following experiments in order to test the action of various classes of substances on the growth of mouse carcinoma. We used in our experiments a mouse carcinoma, transplanted by us through many generations from a spontaneous tumor sent from Granby, Mass., about seven or eight years ago. In this tumor the cells after a certain initial period of growth are arranged in solid alveoli. Later caseous necrosis sets in in the central parts of the tumor. The tumor was inoculated into American mice weighing approximately sixteen grams. The inoculation was positive in 80 to 90 per cent. (on the average in 82 to 83 per cent.) of the animals. In testing the effect of a certain substance on tumor growth, the mice were injected on the 9th, 10th, 11th, and 12th days after inoculation. The large majority of all injections were done intravenously, the veins of the tail being used. In some cases the effect of subcutaneous injections was compared with the intravenous administration. Two methods were used in determining

* Received for publication, July 16, 1914.

[1] The greater part of this work was carried out during the year 1912 and during the first half of 1913. Since then we enlarged the scope of our work by making additional experiments of a quantitative character. Previous references to these experiments are found in the following communications: Loeb, L., and Fleisher, M. S., *Interstate Med. Jour.*, 1912, xix, 476; 1913, xx, 569; *Jour. Am. Med. Assn.*, 1913, lx, 1857.

In some of our previous papers references have been made to the literature on this subject as far as it preceded our own work. Since we have published our first report a number of communications by various authors have appeared which dealt with the effect of isolated substances rather than with a comparative study of various groups of substances which we undertook. A review of the literature on this subject is given by Keysser, F., *Ztschr. f. Chemotherap., Ref.*, 1914, ii, 1325.

the effect of the injections. (1) On the ninth day after inoculation a drawing of each tumor was made; on the twelfth and thirteenth days another drawing was made. This permitted us to determine with a sufficient degree of certainty whether a tumor had grown very much during this period or whether it had been retarded in its growth. This method was sufficient for determining whether or not a certain substance exerted a decided effect on the tumor growth. The method is inadequate for the determination of finer quantitative differences in the growth of tumors. (2) On the ninth day a drawing of the tumor was made. This drawing was compared with drawings of a series of other tumors of known weight, and thus the approximate weight of the tumor on the ninth day was determined. On the twelfth and thirteenth days further drawings of the tumors were made, and on the thirteenth day the tumors were removed and weighed, either each tumor individually or in smaller groups of tumors whose weight on the ninth day had been determined. On this day the increase in weight expressed in per cent. of the original weight during the period of treatment was ascertained. Before stating the effect of the various substances, it will be necessary to inquire into the degree of accuracy of our methods.

TABLE I.

Group No.	No. of tumors.	Calculated weight.	Actual weight.	Per cent. +	Per cent. −
1	21	6,525 gm.	5.717 gm. (+14%)	373%	331%
2	19	3.545 gm.	3.951 gm. (−11.4%)	17%	225%
3	10	2.550 gm.	2.575 gm. (> 1%)	26%	49%
4	15	2,910 gm.	3.377 gm. (14%)	52%	216%
		15.530 gm.	15.620 gm.	+468%	−821%
		−0.57%		−353%	
				Average 5.8%	

The first step in our second method consists in the calculation of the weight of the tumor on the ninth day on the basis of our drawings with the aid of weighed models. We investigated the accuracy of this determination by first calculating on several occasions the weight of a certain number of tumors nine days old by means of

drawings and then immediately removing the tumors and weighing them. We compared the weight of sixty-five tumors obtained first through calculation on the basis of drawings and models and subsequently through actual weighing. We arranged these into four groups according to the period in the course of our experiments at which the tumors were removed; in the first group are put together all those tumors removed in the beginning, at a time when the number of models at our disposal was still relatively small; in the fourth group those removed towards the latter part of our work, when the number of models had increased considerably.

In table I we find in the first column the weight calculated with the aid of drawings and models; in the second column the actual weight as determined with the aid of the balance. There is in the individual groups a maximum deviation of 14 per cent. in both directions, and if we add all the tumors there is found to be a little more than 0.5 per cent. error in the determination of weight by means of drawings and models. If we leave out of consideration the question of weight of the individual tumors and consider merely the means of the errors expressed in percentage of the actual weight, we find that on the average the weight of the individual tumors was underestimated. If we consider the fact that we examined usually two series of tumors at the same time and that therefore the error in both series which we wished to analyze was probably approximately of the same order and that the differences in weight which we produced experimentally were of a considerably higher order than the error of the method, and furthermore that all the experiments we made with this method were confirmatory of each other, we may conclude that the second method used is adequate for our purposes.

The reliability of the first method (palpation) we can test by determining in the same experiments the effect of the treatment by both methods. We first determine the increase in size by palpation and then the increase in weight by the second method. In weighing in a number of experiments each individual tumor, after its relative growth had previously been determined by palpation, and drawing and comparing its size on the thirteenth day with its original size on the ninth day, when the injections were started, we

could approximately come to a decision as to the accuracy of the first method.

TABLE II.

Comparison of Percentage of Growth of Tumors by Weighing and by Palpation.

	Tumors which by means of palpation had been diagnosed as retarded in growth.		Tumors which by means of palpation had been diagnosed as growing normally.	
	No. of mice.	Per cent. of growth (found through weighing).	No. of mice.	Per cent. of growth (found through weighing).
Series A II.				
Colloidal copper controls..	21	26%	14	70%
			In two cases we were uncertain, and both gave less than the average growth.	
Immunized mice.........	3	3%	17	102%
Series C II.				
Normal uninjected controls				
Class d...............	4	47%	11	203%
	In two cases we were uncertain whether retarded or not			
Class f...............	7	134%	28	294%
	In five cases we were uncertain whether retarded or not			
Mice injected with casein				
Class a...............	13	33%	10	97%
Class c...............	8	56%	8	163%
Class d...............	6	117%	4	236%
Class f...............	3	135%	4	291%
Mice injected with aurol				
Class a...............	12	68%	11	132%
Class c...............	4	12%	9	198%
Class d...............	3	−84%	8	202%
Class f...............	2	−54%	1	217%

Table II gives the results of these comparisons. We separated in each group those tumors which by palpation were found to show retarded growth from those showing normal growth, and determined in each the average increase in weight. The differences in actual weight between the two kinds of tumors are marked; and the results obtained by both methods agree, if we consider the various groups of tumors. In individual tumors there may occasionally be found a wrong diagnosis made by means of the first method.

In almost all cases the differences in actual weight between the tumors diagnosed as retarded in growth and growing normally are

more than 100 per cent. We have to consider that we must in each case compare tumors of the same class, inasmuch as tumors grow, as a rule, more rapidly from the ninth to the thirteenth day, the smaller they were on the ninth day; therefore, the tumors of class f grow more rapidly than tumors of class d; tumors of class d grow more rapidly than tumors of class c, and tumors of class c grow more rapidly than tumors of class a; and that therefore in class f a relative increase is regarded as retarded growth which in class a would have been considered as marked growth.

In other experiments we did not weigh each individual tumor, but certain groups of tumors, those tumors being in each case grouped together which had a similar weight on the ninth day. In this case we could less definitely determine how far the results obtained by both methods agreed, but, as far as could be judged, the results agreed fairly well.

In order to eliminate as much as possible the subjective factor in the first method, two persons made in a certain number of cases parallel experiments, using palpation and drawing as a means of determining the growth of the tumors; both persons arrived independently at the same conclusions. Furthermore, as far as the principal problems are concerned, we used both methods and the results obtained by both methods were on the whole confirmatory of each other. We may therefore conclude that the first method is sufficiently accurate to permit us to determine in a general, but not in an exact, quantitative way, whether or not a certain experimental interference had a retarding influence on the tumor growth. In a number of our earlier experiments we used, however, the first method exclusively, and we shall distinguish between results obtained by both methods and those obtained only by the first method.

We examined by these methods and within the time interval stated the action of various classes of substances, distilled water, a number of inorganic salts, a number of inorganic colloidal substances, various organic colloidal and non-colloidal substances, and especially hirudin alone and in combination with some other substances. We limited the treatment in the large majority of cases to four intravenous injections given on four consecutive days, because it was necessary to treat all animals in a comparable way. In ex-

periments on which we shall report in a further communication the four intravenous injections were preceded by a preliminary set of four intravenous injections. It is often difficult to give more than eight intravenous injections to a mouse, and we used, therefore, four injections as the standard for comparison.

In tables III, IV, V, and VI, those of our experiments in which the first method was used are grouped together. We determined first the lethal dose of each substance to be used. The quantity given varied somewhat in the case of the different substances. It was sometimes half the lethal dose, occasionally less, and in the majority of the cases somewhat more than one half the lethal dose. In the second column of our tables the lethal doses are represented, in the third column the dose given in our experiments. The fourth column gives the coefficient of safety; namely, the relation between the lethal and experimental dose. We used two criteria in judging the action of the various substances.

(1) One day after the first injection we examined the tumors, making incisions into them. Some substances exerted a distinct effect on the tumor after one injection. In columns 5 to 9 we gave the result in cases in which these examinations were made. Such examinations one day after the injection were not made in all the substances. We called "much affected" tumors those in which on the tenth day a large part of the growth was found to be necrotic and softened and sometimes hemorrhagic in the central portion. Some living tumor tissue may still be found in the periphery. In tumors which are designated as "partly affected" there is still a slight softening in the center of the tumor, but at least one half of the peripheral portion is not affected. In tumors "slightly affected" there is a very small area of softening in the central portion of the tumor, but most of the tumor appears normal.

(2) In columns 10, 11, and 12, we give the result of the effect of four injections on tumor growth, the figures showing the number of tumor mice observed. "Retarded growth" shows those tumors which grew distinctly less than the average tumor of a certain size. A certain number of tumors became distinctly smaller under the influence of the treatment. They are marked "retrogressing." It happens, of course, occasionally that a tumor retrogresses spon-

taneously, especially a tumor of small size; but this is on the whole not a usual occurrence. Only those mice were considered which lived through the whole period of the experiment, while those, for instance, which died on the fourth day were not included.

A number of the substances were obtained from manufacturers; others were prepared in our laboratory by Professor E. H. Keiser. Colloidal copper and platinum were prepared in accordance with Bredig's method.[2] Various organic substances were prepared according to the ordinary methods of biological chemistry.[3]

If we now consider the results in table III, we find that intravenous injection of distilled water did not retard the growth of tumors, neither did one injection cause any noticeable changes in the tumors. Two sulphur preparations (milk of sulphur, and hydrochloric acid plus sodium thiosulphate) were examined after one injection. No effect was noted. Various inorganic salts (copper nitrate, copper ammonium sulphate, mercury bichloride, gold sodium chloride (Merck), sodium tartrate, lanthanum nitrate) did not show any

[2] Some other substances were prepared in the following manner:

Copper Casein B.—Add a concentrated solution of $CuSO_4$ to an equal amount of a saturated solution of casein (dissolved in NaOH). Dissolve the precipitate in NH_4OH and precipitate with absolute alcohol.

Copper Casein C.—Dissolve copper tartrate in NaOH and add to an equal quantity of saturated casein solution (dissolved in NaOH). Precipitate in absolute alcohol.

$H_2S + NaNO_2$.—Pass H_2S through a moderately strong $NaNO_2$ solution; this gives a yellow precipitate. Dilute with H_2O. After standing for twenty-four hours the precipitate is dissolved.

Sulphur.—Colloidal (chemically prepared). Heat flowers of sulphur with NaOH and a little water; boil and pass steam through a condenser. This gives a milky liquid with slight reddish color.

Selenium.—Colloidal (chemically prepared). Dissolve the selenium by boiling with concentrated HNO_3, and evaporate this to dryness. Heat and sublime the SeO_2. Dissolve the crystals in H_2O and then run in H_2S. This gives an orange colored liquid.

Casein and Sulphur.—Dissolve 10 gm. of casein (purified) in 600 c.c. of distilled water to which 20 c.c. of normal NaOH have been added. Pass a stream of H_2S through this for from twelve to twenty-four hours, until saturated with H_2S. This gives an acid reaction to litmus. Then draw air through until the H_2S is no longer perceptible.

[3] Protamin was prepared according to Kossel's method from dried salmon milt, which we obtained through the kindness of Professor Alonzo E. Taylor, of the University of Pennsylvania.

TABLE III.

Substance.	Lethal dose.	Usual dose.	Coefficient of safety. Lethal dose: usual dose.	One injection.				Several injections.		
				Much affected.	Partly affected.	Slightly affected.	Normal.	Retarded growth.	Retrogressing.	Normal growth.
Copper nitrate m/250......	0.6 c.c.	0.4 c.c.	1 : 0.66	—	—	—	—	2	—	25
Copper ammonium sulphate 0.7%................	—	0.3 c.c. 0.21 mg.	—	—	—	—	—	1	—	10
Mercury bichloride m/400..	0.4 c.c.	0.25 c.c.	1 : 0.62	—	—	—	—	—	—	5
Lanthanum nitrate........	4.0 mg.	2.5 mg.	1 : 0.62	—	—	—	—	—	—	3
Sodium tartrate 3⅓%.....	0.5 c.c. 17.0 mg.	0.4 c.c. 14.0 mg.	1 : 0.8	—	—	—	—	—	—	15
Gold potassium cyanide (Merck)..............	0.15 mg.	0.05 mg.	1 : 0.33	—	—	—	—	5	—	17
Gold sodium chloride (Merck)..............	0.8 mg.	0.4 mg.	1 : 0.5	—	—	—	—	1	—	20
Distilled water...........	—	1.0 c.c.	—	—	—	—	3	2	—	19
Milk of sulphur...........	0.75 c.c.	0.37 mg. 0.16 c.c.	1 : 0.5– 0.25	—	—	—	3	—	—	—
Hydrochloric acid + sodium thiosulphate	0.75 c.c.	0.5 mg. 0.25 c.c.	1 : 0.66– 0.33	—	—	—	7	—	—	—

TABLE IV.

Substance.	Lethal dose.	Usual dose.	Coefficient of safety. Lethal dose: usual dose.	One injection.				Several injections.		
				Much affected. (1)	Partly affected. (2)	Slightly affected. (3)	Normal.	Retarded growth.	Retrogressing.	Normal growth.
Colloidal copper.........	2.5 c.c. +	1 c.c.	1 : 0.4–	—	—	1	18	371	—	277
Colloidal copper hydrate (Mulford)............	0.1 c.c.	0.05 c.c.	1 : 0.5	—	—	—	—	2	1	20
Colloidal copper (Heyden) 0.3%................	0.7–0.4 c.c. 2.1–1.2 mg.	0.25 c.c. 0.75 mg.	1 : 0.35– 0.62	15	—	1	2	10	—	4
Colloidal platinum........	2.5 c.c. +	1.0 c.c.	1 : 0.4–	—	—	—	—	12	—	3
Colloidal sulphur (electrolytic)................	—	1.0 c.c.	—	—	—	—	—	3	—	7
Colloidal sulphur (chemical)..................	—	1.0 c.c.	—	—	—	—	—	1	—	10
Colloidal selenium (Mulford)................	0.1 c.c. 0.025 c.c.	0.0125 c.c.	1 : 0.125– 0.5	—	—	—	—	6	—	15
Colloidal selenium (prepared by us chemically).	—	1.0 c.c.	—	—	—	—	—	—	—	11
Aurol (Hille) 15%........	0.7 c.c.	0.5 c.c.	1 : 0.72	—	—	—	—	14	7	29
Copper casein A.........	1.2 mg.	1.0 mg.	1 : 0.83	—	—	—	—	8	—	3
Copper casein B.........	1.2 mg.	1.0 mg.	1 : 0.83	—	—	—	—	—	—	9
Copper casein C.........	12 mg.	10.0 mg.	1 : 0.83	—	—	—	—	11	—	—

noticeable inhibiting effect on tumor growth. Although the number of tumor mice treated was not great in the case of each individual substance, the number is sufficiently large (eighty-three) if we add animals used in the various experiments. We may therefore conclude that inorganic salts injected under the conditions of our experiments had not a sufficiently marked retarding influence on tumor growth to be accessible to determination by our first method. This does not exclude the possibility that through our second method a slight influence might be found. Only in the case of gold potassium cyanide was there possibly a slightly retarding influence present, but if present at all, it certainly was not marked.

In table IV some inorganic colloidal substances and combinations between copper and casein are considered. Besides the colloidal copper prepared by us electrolytically in accordance with Bredig's method we used a preparation of colloidal copper made by the H. K. Mulford Company, and one by Heyden (Dresden). The exact composition of these two preparations is not known to us. Colloidal platinum we prepared ourselves in accordance with Bredig's method. Colloidal sulphur we prepared either chemically or by the electrolytic method of Svedberg. Aurol (Hille) is a preparation of colloidal gold which was sent us by the Vitochemical Laboratories, Chicago.[4] Professor Keiser prepared several combinations of copper and casein (B and C).

The results are as follows: Colloidal copper prepared electrolytically, colloidal copper (Heyden), colloidal platinum, aurol (Hille), and two of the copper casein preparations had a distinct retarding influence on tumor growth; while the colloidal copper hydrate (Mulford), colloidal sulphur prepared chemically, colloidal selenium prepared by us chemically, and one copper casein preparation (B) were without decided influence. It is possible that the copper casein preparation B had undergone secondary changes at the time we used it and that this fact may account for the negative results. In the case of the electrolytically prepared sulphur and colloidal selenium (Mulford) there was perhaps a slight effect present, but it was certainly not marked. It is possible that small quantities of colloidal

[4] The H. K. Mulford Co. and the Vitochemical Laboratories kindly put their preparations at our disposal for experimental purposes.

platinum were admixed to the solution of electrolytically prepared sulphur and that this may account for a slight effect which was possibly present.

Our method does not permit us to differentiate quantitatively between the efficacy of the various active substances. We observed a temporary retrogression during the period of injection in a few animals treated with aurol; but the number of retrogressing tumors in this case is too small to be of much significance. On the whole, however, the difference in the efficacy of the various preparations does not seem to be striking. Two preparations were tested as to their early effect on the tumor after one injection. Our own preparation of colloidal copper was without effect in this respect, while the Heyden preparation of colloidal copper had a marked effect.

We may then conclude that in contradistinction to the inorganic salts of molecular dispers character, which are without marked effect, certain colloidal solutions of heavy metals (copper, platinum, gold) are effective, inasmuch as they retard definitely the growth of a number of tumors of injected animals; of a similar activity are also combinations of copper salts and casein. However, according to the method of preparation of some of these substances their efficacy differs. None of these substances has a markedly destructive action on mouse tumors. In the majority of cases the effect is transitory and does not lead to a cure.

In table V the results of the injections of various organic substances are represented. We used various albuminous substances, as well as starch and lecithin, as representative of the carbohydrates and lipoids. In addition we used horse serum; tuberculin we used as a representative of bacterial toxins. Ethylhydrocuprein was successfully used by Morgenroth[5] in the treatment of pneumococcus infections. Ergamin is a substance producing symptoms somewhat resembling anaphylactic shock. Of all these substances only casein and nucleoproteid preparations had a sufficiently retarding effect on tumor growth to be demonstrable by our method. And the effects of these substances seemed to be on the whole of a similar order to those produced by the colloidal metals. They differ, however, from

[5] We are indebted to Professor Morgenroth and the manufacturers for the material at our disposal.

TABLE V.

Substance.	Lethal dose.	Usual dose.	Coefficient of safety. Lethal dose: usual dose.	One injection. Much affected.	One injection. Partly affected.	One injection. Slightly affected.	One injection. Normal.	Several injections. Retarded growth.	Several injections. Retrogression.	Several injections. Normal growth.
Ethylhydrocuprein 0.375% ...	0.4 c.c. 1.5 mg.	0.25 c.c. 0.94 mg.	1 : 0.62	—	—	—	—	—	—	4
Ergamin (Burroughs and Wellcome).....	—	1.0 mg.	—	—	—	—	—	—	—	6
Starch 5% suspension.......	1.0 c.c. +	0.75 c.c.	1 : 0.75+	—	—	—	11	—	—	6
Lecithin 0.1% (Merck ovo)...	0.75 c.c. 0.75 mg.	0.5 c.c. 0.5 mg.	1 : 0.66	—	—	—	6	—	—	6
Pepton 5%	1.5–1.25 c.c. 0.1–0.075 mg.	0.6 c.c. 0.03 mg.	1 : 0.4–0.5	—	—	—	13	101	—	21
Protamin 0.1% .	0.7 c.c. 0.7 mg.	0.5 c.c. 0.5 mg.	1 : 0.72	—	—	—	10	—	—	10
Serum globulin 15%..........	2.0–1.5 c.c. 0.3–0.22 gm.	1.0 c.c. 0.15 mg.	1 : 0.5	—	—	—	23	—	—	9
Ovalbumin saturated	1.5–1.0 c.c. 0.37–0.25 gm.	0.75 c.c. 0.18 gm.	1 : 0.5–0.75	—	—	—	102			23
Tuberculin (AT) 50%..........	0.65–0.2 c.c.	0.5–0.15 c.c.	1 : 0.8–0.75	—	—	—	14	—	—	9
Horse serum.....	1.5 c.c.	1.0 c.c.	1 : 0.66	—	—	—	20	—	—	4
Casein 1⅔%.....	2.0 c.c. 32.0 mg.	1.0 c.c. 16.0 mg.	1 : 0.5	46	6	20	22	68	—	54
Casein 1⅔% + sulphur.......	2.0 c.c. 32.0 mg.	1.0 c.c. 16.0 mg.	1 : 0.5	66	15	23	3	34	—	16
Nucleoproteid (lymph gland of cattle) 15%....	0.75–0.25 c.c. 113.0–38.0 mg.	0.5–0.15 c.c. 76.0–22.0 mg.	1 : 0.66	18	1	4	28	7	—	5
Nucleoproteid (kidney of cattle) 15%......	0.25–0.15 c.c. 38.0–22.0 mg.	0.2–0.1 c.c. 30.0–15.0 mg.	1 : 0.8–0.66	10	2	5	13	17		9

the electrolytically prepared colloidal copper through the edema or hemorrhages which they cause and which is noticeable twenty-four hours after the first injection in many tumors of the injected mice. Here again casein and nucleoproteid were the only active substances, while all the other substances in table V were without direct effect on the tumors.

In table VI the effect of hirudin and various combinations of hirudin and other substances is given. On the whole, hirudin is, as far as the number of affected tumors is concerned, not more effective than colloidal copper. It differs, however, from colloidal copper in

TABLE VI.

Substance.	Lethal dose.	Usual dose.	Coefficient of safety. Lethal dose: usual dose.	One injection.				Several injections.		
				Much affected.	Partly affected.	Slightly affected.	Normal.	Retarded growth.	Retrogression.	Normal growth.
Hirudin 0.25% solution	1.5–0.1 c.c. 3.7–0.25 mg.	0.05–1.0 c.c. 2.5–0.125 mg.	1 : 0.5–0.065	11	—	—	—	114	70	102
Hirudin + nucleoproteid	—	Usual dose of hirudin + usual dose of nucleoproteid		10	—	—	—	1	11	2
Colloidal copper + hirudin	—	Colloidal copper 0.5–0.25 c.c. Hirudin 0.125–0.25 mg.		—	—	—	—	11	24	6
Different one each day: colloidal copper, casein, hirudin, nucleoproteid	—	Usual doses		—	—	—	—	34	—	12
Colloidal copper, intravenously. Hirudin, subcutaneously	—	Colloidal copper 1.0 c.c. Hirudin 0.80 mg.		—	—	—	—	7	—	6
Hirudin, subcutaneously 0.25%		0.25 c.c. 0.8 mg.		—	—	—	—	2	—	9

two respects: (1) the number of tumors retrogressing under the influence of hirudin is considerably greater; and (2) one single injection has a marked effect on the tumor, causing hemorrhage and edema. As we saw previously, colloidal copper did not have such an effect. The retrogression of the tumors is usually not a permanent one; but after temporary cessation of the injections the tumors begin in the majority of cases sooner or later to grow again; a certain number of the tumors, however, retrogress definitely. Hirudin

given subcutaneously was without a marked effect. It is, however, to be noted that the efficacy of several samples of hirudin differed and that the results obtained at different periods were not equally marked. More effective than hirudin alone was a combination of hirudin and either nucleoproteid or colloidal copper, both substances being given at the same time. In combination with nucleoproteid the number of retrogressions was considerably greater than in cases in which hirudin alone was used. It is, however, to be considered that at that time the hirudin which we used was stronger than some later samples that we received. A combination of colloidal copper and hirudin was also effective. The number of retrogressing tumors was here very great, much greater than with either hirudin or colloidal copper alone. This combination is also highly toxic and a large number of the tumor mice injected died. If the hirudin was given subcutaneously instead of intravenously, while the colloidal copper was given intravenously, the effect was merely that of the colloidal copper. Hirudin administered subcutaneously was therefore again ineffective.

As we shall see later, processes of immunization take place in the course of a series of injections. It was therefore of interest to test the effect of a combination of four different substances, a new substance being given on each successive day. Colloidal copper, casein, hirudin, and nucleoproteid were thus alternately administered. There was, perhaps, a somewhat stronger effect than in cases in which each of these substances was given alone for four successive days, but the difference was not marked. This result points to the conclusion that one single injection of a certain substance does not lead to the production of a noticeable degree of immunity.

In addition to these experiments carried out by means of the first method, we carried out a series of experiments with the second method, in order to obtain results of a more quantitative character.

All the tumors treated in an experiment in the same manner represented a series. Inasmuch as the percentage increase of tumors in weight or volume depends on their weight at the time when the experiment was started, we had to subdivide a series in different classes, according to the weight of the tumors on the ninth day. In

all experiments the classes were as follows: class a = tumors weighing 300 milligrams or more; class c = tumors weighing between 230 and 300 milligrams; class d = tumors weighing between 130 and 230 milligrams; class f = tumors weighing between 25 and 130 milligrams.

In cases in which we were unable to weigh each tumor at the end of the experiment, we divided the various classes into smaller groups, according to the weight of the tumors on the ninth day, and all the tumors of one group were weighed together. In table VII the results obtained by the second method are given.

In the first column we find the number of mice used in testing each substance. In the case of pepton and ovalbumin we referred separately to the individual experiments made with each of these substances. In addition we give a summary of the three experiments done with each of these two substances. In the third experiment we give two sets of figures; in one set all the tumors were included; in the corrected set we omitted from consideration the various retrogressing tumors. This seemed to be a fairer way of calculation, because in this experiment there were also a number of retrogressions in the control tumors. In all our tables only the tumors of such animals were included as lived to the end of the experiment. All animals that died before that time were omitted. The last four columns of the table (a, c, d, f) give the percentage increase in weight in the different classes of tumors arranged in accordance with their weight nine days after inoculation; a represents the largest, f the smallest tumors. Columns 2, 3, 4, and 5 (α, β, γ, δ) give the average percentage increase of the tumors of a series. We describe in the following paper[6] on "Immunization against substances inhibiting tumor growth" the methods used by us for the determination of these averages.

In the first line in table VII the percentage increase of normal control mice is given. The second line gives the corresponding figures for mice injected with colloidal copper. We recognize clearly the depressing effect of colloidal copper on tumor growth. While the normal control tumors gain 170 to 180 per cent. in weight in four days, in the tumors of the injected animals, including those in

[6] Fleisher, M. S., Vera, M., and Loeb, L., *Jour. Exper. Med.*, 1914, xx, 522.

TABLE VII.

	No. of mice.	α	β	γ	δ	a	c	d	f
Normal controls	262	177%	192%	174%	180%	117%	140%	181%	251%
Colloidal copper	300	103%	107%	110%	107%	81%	81%	123%	155%
Aurol	50	111%	112%	97%	106%	[23]98%[7]	[13]137%	[11]141%	[8]11%
Hirudin	69	139%	150%	147%	145%	—	[7]126%	[27]150%	[35]166%
Casein	56	84%	112%	138%	111%	[23]61%	[15]104%	[10]158%	[7]228%
Pepton 1	42	111%	154%	131%	121%	[8]60%	[5]145%	[13]132%	[21]185%
Pepton 2	52	191%	192%	183%	189%	[3]135%	[4]195%	[17]220%	[28]181%
Pepton 3	28							[14]157%	[14]108%
Corrected, omitting retrogressing tumors similar to those in controls	22							[12]177%	[10]213%
Pepton. Summary	122	152%	173%	157%	155%	94%	170%	169%	158%
Pepton. Summary. Corrected figures	116							176%	193%
Ovalbumen 1	39	185%	174%	176%	177%	[1]120%	[2]236%	[14]182%	[22]166%
Ovalbumen 2	48	166%	192%	159%	172%	[4]99%	[6]123%	[15]182%	[23]233%
Ovalbumen 3	53	145%	161%	157%	154%	[8]99%	[14]134%	[14]118%	[17]277%
Corrected, omitting retrogressing tumors	46	160%	205%	191%	185%	[7]120%	[13]145%	[12]176%	[14]325%
Ovalbumen. Summary	140	165%	176%	164%	168%	106%	164%	161%	225%
Ovalbumen. Summary. Corrected figures	133	170%	191%	175%	178%	113%	169%	180%	241%

[7] The small figures at the upper left side of some of the percentage figures in the last four columns indicate the number of mice used in the various experiments.

which no effect was noticeable, as well as those which were influenced by the colloidal copper, the increase was from 100 to 110 per cent.

As will be seen from the tables, our figures in the case of colloidal copper are based on the examination of a large number of mice.

Aurol is effective, but probably less so than colloidal copper. In this experiment some of the smaller tumors retrogressed and the figure for f is therefore very low. The low percentage increase in group f reduces considerably the average increase of the tumors treated with aurol. Inasmuch as the number of mice in class f was very small, we must not attach too much importance to this figure. In the other classes the reduction in percentage increase in weight was considerably less than in colloidal copper. In the case of aurol we noticed a dark bluish grey discoloration of the tumors, liver, and occasionally the spleen, after four injections had been given. Apparently this discoloration was due to a deposit of foreign material in capsule and stroma.[8]

Hirudin also had a distinctly inhibiting effect. The full effect can, however, not be appreciated from a study of our figures, as there was much coagulated blood and marked edema in the tumors of the injected mice. The effect of hirudin is therefore more marked than is indicated by the figures of our table. Besides, a number of these tumors are usually found to retrogress when the observation of the tumors of mice injected with hirudin is prolonged.

Casein is also effective, but perhaps not quite so effective as colloidal copper. Here the average increase determined according to the methods is especially low because the number of the large tumors was great and their growth was unusually retarded.

While casein served as a representative of the albuminous substances which we had found effective in employing the first method of measurement, we used pepton and ovalbumin as representatives of those proteids which the first method showed to be ineffective, or less effective. With each of these two substances we made three experiments. In the first experiment the mortality among the in-

[8] We intend to examine the character of the deposit more closely as soon as possible. On fixed and stained specimens the cause of the discoloration could not be determined.

jected mice had been very great, about one half of all the injected mice dying after the first injection. The remaining mice also were somewhat affected. The mice injected with pepton showed very much retarded tumor growth. The effect was here similar to colloidal copper. The mice injected with ovalbumin behaved differently in different classes. In this experiment the first dose of the substances given had evidently been too strong and the general health of the mice may have suffered in consequence. We may state that neither after injection of colloidal copper nor of casein does the general condition of the mice seem to suffer.

In the second experiment an effect on the part of pepton as well as ovalbumin was either absent or very small. A slight reduction in class f must not be overestimated, inasmuch as in class f spontaneous retrogressions are apt to occur. In the third experiment there occurred in the control experiments a number of spontaneous retrogressions of tumors. Retrogression of tumors in the animals injected with pepton or ovalbumin must therefore in the main not be attributed to the action of these substances, and the mice with retrogressing tumors should be entirely omitted, in order to obtain a fair estimate of the action of these substances. With these corrected figures ovalbumin was without effect in the third experiment, while the original figures indicated some effect of ovalbumin, which was, however, considerably less than casein. With pepton only tumors of classes d and f were used in this experiment. There was in this case some effect in class f noticeable in using either of the two figures. The corrected figures of class d were approximately the figures of normal mice, while the uncorrected figures were similar to those of casein. If we consider the summary of the three experiments we find that pepton has decidedly less effect on the tumors than casein, although it has some effect, while ovalbumin is almost without effect. These experiments make it probable, therefore, that ovalbumin and pepton are decidedly less effective than casein, although they may have some effect, especially pepton; and they confirm therefore, on the whole, the conclusions based on the first method. We believe, however, that further experiments are necessary to make our conclusion concerning the different efficacy of various proteids definite.

While in the case of most substances which we used we studied the effect on the growth between the ninth and thirteenth day after the inoculation of the tumor, we determined in the case of colloidal copper and of hirudin in addition the effect of injections given from the second to the sixth day after inoculation. We found that the tumors of animals treated in this way were on the ninth day after inoculation as large as tumors of animals which had not been injected. It seems, therefore, that injections given at an early date are without a noticeable effect on tumor growth. This is perhaps due to the fact that at the early period the vascularization of the tumor has not yet progressed as far as later, and perhaps also to the lesser interference with the circulation through pressure at this early period. If, however, the tumors have reached a certain development, such as is found between the ninth and twelfth days after inoculation, they are much more accessible to the action of colloidal copper.

TABLE VIII.

Decrease of Percentage Increase in Weight under the Influence of Colloidal Copper in the Different Classes.

Group.	Tumors. Control mice.	Increase in weight of tumors in mice injected with copper.
a	100 %	69 %
c	100 %	58 %
d	100 %	68 %
f	100 %	62 %

It was of interest to determine whether there was any difference between small and large tumors in the degree with which they are influenced by the injection of colloidal copper. In table VIII the tumors are arranged in classes, a, c, d, f, according to their size. If we take 100 per cent. as the standard of the average increase in weight of the various classes of control tumors, the tumors in the various classes of injected mice lose approximately in the same proportion under the influence of colloidal copper. Tumors of different size are therefore relatively influenced in a similar manner through the injections of colloidal copper. While the relative loss in all classes is approximately the same, the absolute loss is of course greater, the greater the percentage increase in weight of the different classes in normal control mice.

SUMMARY.

We have described the methods used in determining the influence of certain substances on tumor growth, and we measured approximately the degree of reliability of the quantitative method used. We examined with these methods various classes of substances,—distilled water, a number of inorganic salts, inorganic colloidal substances, various organic colloidal and non-colloidal substances, especially various proteids, tuberculin and hirudin alone as well as in combination with other substances. Distilled water, various inorganic sulphur preparations, and various inorganic salts did not show an inhibiting effect on tumor growth sufficient to be detected by means of our first method. Only in the case of gold potassium cyanide was there possibly a slightly retarding influence present. On the other hand, certain colloidal solutions of heavy metals (copper, platinum, gold) retard the growth of a number of tumors of injected animals. Certain combinations of copper salts and casein act in a similar manner. Of the organic substances used, casein, nucleoproteid, and hirudin were active, while the other proteids tested, as well as various other organic substances and tuberculin and lecithin, seemed to be either without effect or weaker than the other substances mentioned as retarding the tumor growth. Hirudin was active and caused in addition to its inhibiting influence the retrogression of a certain number of tumors. Especially active was a combination of hirudin with colloidal copper and of hirudin with nucleoproteid.

One single injection of casein or nucleoproteid, or of the Heyden preparation of colloidal copper, leads to a more or less marked edematous condition of a certain number of tumors, while hirudin caused in addition, in many cases, marked hemorrhages in or around the tumors. Other substances which we tested did not show this effect, although their inhibiting action on tumor growth may have been equally strong. Very young tumors (two to six days old) are not retarded in their growth through injection of colloidal copper or hirudin, while nine to thirteen days old tumors are, independently of their size on the ninth day, inhibited in approximately the same relative degree; absolutely, however, the more rapidly growing smaller tumors are more markedly inhibited than the normally more slowly growing larger tumors.

IMMUNIZATION AGAINST THE ACTION OF SUBSTANCES INHIBITING TUMOR GROWTH.*

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Theoretical as well as practical considerations suggested to us the line of investigations that is reported in this communication. One of us[1] has formerly shown that through the action of heat or chemical injurious influences to which the tumor cells are subjected before transplantation it is possible to decrease the virulence of the resulting tumors. It was of interest to inquire to what extent this peculiarity of growth might be inherited by new generations of tumor cells, and one of us[2] investigated, therefore, the resistance to heat of particles of tumor which had developed in animals from tumor particles exposed to a certain degree of heat before transplantation.

We found with the method used at that time that neither a summation of the effects of successive exposures to heating nor an immunity against the effects of heat can be demonstrated under these conditions. We also found that after transplantations of particles of tumors which developed after previous heating, the new tumors grew as well as control tumors obtained through transplantation of particles from previously unheated tumors. Notwithstanding these negative results the problem seemed to us of sufficient importance to justify further attempts on the basis of the

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[1] Loeb, L., *Virchows Arch. f. path. Anat.*, 1903, clxxii, 345; *Am. Med.*, 1905, x, 265.

[2] Corson-White, E. P., and Loeb, L., *Centralbl. f. Bacteriol., 1te Abt., Orig.*, 1910, lvi, 325.

results communicated in the preceding paper.[3] We have shown that a number of different substances exert a definite inhibiting action on the growth of mouse tumors. The opportunity presented itself to investigate whether an immunity or increased resistance[4] can be produced through a series of preliminary injections, and whether such an immunization depends on processes called forth in some organ or tissue of the injected animal or in the tumor cells proper.

There was a further reason for undertaking these investigations. In a certain number of cases we observed in patients suffering from cancer that at first the intravenous injections of colloidal copper had an undoubted effect on the tumor, causing diminution in the size of the ulcerated area and partial shrinking of the tumor or an arrest in the progress of the disease which had been rapid before. But these effects were temporary and after a certain time the disease began again to progress. This observation could be explained by the assumption of processes of immunity against the injected substance taking place either in the injected organism or in the tumor cells, a possibility which we had indeed considered from the beginning of our experiments.

In order to test the existence of such processes of immunity we proceeded in the following manner: We injected intravenously a series of animals with colloidal copper from the ninth to the thirteenth day after transplantation of the tumor, and we determined the growth of these tumors. In a second series we injected animals from the second to the fifth day after inoculation with colloidal copper and again from the ninth to the thirteenth day. We had

[3] Loeb, L., and Fleisher, M. S., *Jour. Am. Med. Assn.*, 1913, lx, 1857. In a different manner Marie, Clunet, and Raulot-Lapointe recently observed a hereditary transmission of a characteristic acquired by sarcomatous cells of the mouse under the long continued influence of Roentgen rays. Under these conditions giant cells, which were absent in the original sarcoma, began to develop and this peculiarity was also noticeable in the following generations of transplanted tumor, although these had not been exposed to the Roentgen rays. (Marie, P., Clunet, J., and Raulot-Lapointe, G., *Bull. de l'Assoc. franç. p. l'étude du cancer*, 1911, iii, 166; abstracted in *Ztschr. f. Krebsforsch.*, 1913, xii, 93.)

[4] We do not attempt to distinguish between partial or total immunity and increased resistance. There do not exist as far as we are aware any sharp criteria which permit a differentiation between two conditions corresponding to these two terms.

previously found that injections of colloidal copper given from the second to the fifth day had no noticeable influence on the tumor growth. Inoculated animals receiving these early injections showed on the ninth day tumors of approximately the same size as animals not receiving these early injections. It was now of interest to inquire whether the early injections which failed to have an inhibiting effect might to a certain extent immunize the animals against the effect of the second set of injections given from the ninth to the thirteenth day. If this should be the case, the injection given from the ninth to the thirteenth day should have less of an inhibiting effect in previously injected animals than in control animals which had not had the preliminary set of injections. We kept another set of control animals which did not receive any injections. If an immunizing effect of the first set of injections should be found to exist, it was furthermore necessary to inquire whether the processes of immunity were restricted to reactive changes in injected animals or in the tumor cells.

This was determined in the following way: A series of animals received, after inoculation with the tumor, two sets of injections of colloidal copper; namely, from the second to the sixth and from the ninth to the thirteenth days after inoculation. Two days after the last injection of colloidal copper, several tumors were extirpated and used for inoculation in a new series of mice. Another series of mice was inoculated with tumor material from mice which had not previously received injections of colloidal copper. Especial care was taken that in both series tumors of approximately similar growth energy were used for transplantation. Both series of mice were then injected with colloidal copper from the ninth to the thirteenth day. If the tumor cells, derived from tumors which had grown in animals injected with colloidal copper, should prove decidedly more resistant towards the effect of the injections of colloidal copper, we may conclude that the tumor cells have been to some extent immunized against the effect of colloidal copper. As controls we kept another series of mice previously inoculated with tumor without injections. We further gave to one series of mice two sets of injections of hirudin, the first set from the second to the sixth day and the second from the ninth to the thirteenth day, and

to another series we gave only the second set of hirudin injections, in order to determine whether also in the case of hirudin an immunity could be produced through previous injections. Just as in the case of colloidal copper, injections of hirudin given from the second to the sixth day do not influence the development of tumors to any marked extent; on the ninth day such tumors are about as large as tumors of animals not previously injected.

In order to determine the effect of the injections we employed in a number of these experiments both methods, described by us in the preceding paper,[5] determining the growth of the tumors on the ninth day by palpation and drawing, and afterwards on the thirteenth day again determining the growth of the tumors by drawing and palpation and then removing the tumors and weighing them either individually or in groups of tumors which were so arranged that tumors united in the same group had about the same size on the ninth day. In addition we carried out other experiments in which we used merely the first method, in order to corroborate the results obtained by the more accurate second method. We made use of the first method in order to determine whether a partial immunity towards the effects of hirudin is located in the tumor cells and is transmitted to following cell generations in a similar manner as in the case of colloidal copper. We furthermore injected a series of animals with colloidal copper or hirudin before inoculation with the tumor in order to determine whether through the injections reactive processes are set up in the organism of the animal, independently of the processes in the tumor cells. Furthermore, by means of the first method we tested the specificity of the immunity produced, giving in the first set of injections, from the second to the sixth day, colloidal copper or hirudin, and in the second set, from the ninth to the thirteenth day, the substance not used in the first set of injections. In this way we could determine whether injections of colloidal copper immunize against the effect of hirudin and *vice versa*. We also tested in a similar manner whether injections of hirudin or colloidal copper, given in one generation of animals, immunize the tumor cells against the substance not used in the first generation in the following generation of animals after transplantation of the treated tumor.

[5] Fleisher, M. S., and Loeb, L., *Jour. Exper. Med.*, 1914, xx, 503.

TABLE I.

Control (uninjected) mice. R	Control (uninjected) mice. B	Mice injected with colloidal copper 9th–13th dy. R	Mice injected with colloidal copper 9th–13th dy. B	Mice injected with colloidal copper 2d–6th and 9th–13th dys. R	Mice injected with colloidal copper 2d–6th and 9th–13th dys. B	Mice inoculated with tumor from mice previously injected with colloidal copper. Injected 9th–13th dy. R	Mice inoculated with tumor from mice previously injected with colloidal copper. Injected 9th–13th dy. B
H 53 mice		A1 24 mice				A1 51 mice	
α 174%	2 m.a. + 113%	α 63% ●	5 m.c. + 48%			α 142% ●	18 m.c. + 100%
β 194%	10 m.c. + 143%	β 121% ●	9 m.d. + 102%			β 282% ●	12 m.d. + 182%
γ 166%	18 m.d. + 147%	γ 85% x	10 m.f. + 110%			γ 178% x	21 m.f. + 451%
δ 178%	23 m.f. + 259%	δ 96%				δ 201%	f.x. + 300%
D 83 mice		A2 34 mice		A2 18 mice			
α 174%	4 m.a. + 96%	α 69% ●	6 m.c. + 40%	α 110% ●	5 m.c. + 74%		
β 177%	8 m.c. + 144%	β 29% ●	5 m.d. + 52%	β 126% ●	7 m.d. + 123%		
γ 153%	35 m.d. + 218%	γ 49% x	23 m.f. + 22%	γ 128% x	6 m.f. + 172%		
δ 168%	36 m.f. + 155%	δ 49%		δ 120%			
B1 19 mice		B1 59 mice		B1 60 mice			
α 164%	1 m.a. + 95%	α 104%	17 m.a. + 76%	α 127%	12 m.a. + 97%		
β 201%	6 m.c. + 116%	β 88%	30 m.c. + 67%	β 140%	26 m.c. + 121%		
γ 179%	6 m.d. + 192%	γ 127%	8 m.d. + 144%	γ 151%	14 m.d. + 171%		
δ 181%	6 m.f. + 312%	δ 106%	4 m.f. + 220%	δ 139%	8 m.f. + 217%		
B2 39 mice		B2 59 mice				B2 62 mice	
α 176%	10 m.a. + 156%	α 83%	10 m.a. + 52%			α 139%	9 m.a. + 103%
β 198%	6 m.c. + 155%	β 138%	14 m.c. + 103%			β 175%	22 m.c. + 106%
γ 187%	5 m.d. + 183%	γ 134%	12 m.d. + 171%			γ 177%	18 m.d. + 231%
δ 187%	18 m.f. + 255%	δ 118%	13 m.f. + 211%			δ 160%	13 m.f. + 269%
C1 18 mice		C1 58 mice		C1 55 mice			
α 138% ●	3 m.a. + 126%	α 125%	14 m.a. + 98%	α 209%	4 m.a. + 187%		
β 143% ●	12 m.c. + 140%	β 150%	15 m.c. + 107%	β 230%	16 m.c. + 172%		
γ 182% x	3 m.d. + 184%	γ 149%	14 m.a. + 172%	γ 223%	19 m.d. + 236%		
154%		δ 141%	15 m.f. + 218%	δ 221%	16 m.f. + 296%		

TABLE I.—*Concluded*.

	Control (uninjected) mice.		Mice injected with colloidal copper 9th–13th dy.		Mice injected with colloidal copper 2d–6th and 9th–13th dys.		Mice inoculated with tumor from mice previously injected with colloidal copper. Injected 9th–13th dy.	
	R	B	R	B	R	B	R	B
C2	50 mice		66 mice				68 mice	
α	220% ●	15 m.d. + 162%	113%	8 m.a. + 98%			204%	6 m.a. + 160%
β	237% ●	35 m.f. + 272%	117%	20 m.c. + 118%			232%	22 m.c. + 157%
γ	174% x		114%	15 m.d. + 95%			222%	22 m.d. + 274%
δ	210%		115%	23 m.f. + 147%			219%	18 m.f. + 296%
	262 mice		300 mice		133 mice		181 mice	
α	177%	a + 117%	103%	a + 81%	163%	a + 142%	200%	a + 131%
β	192%	c + 140%	107%	c + 81%	165%	c + 122%	229%	c + 121%
γ	174%	d + 181%	110%	d + 123%	167%	d + 176%	189%	d + 229%
δ	180%	f + 251%	107%	f + 155%	165%	f + 228%	206%	f + 339%

α Percentage of increase on comparing the sum of the original weight of all tumors (calculated) with the sum of the end weight (weighed).

β Multiply the average percentage of increase of each class (a, c, d, f) by the number of mice in that class, add the figures thus obtained, and divide the sum by the total number of mice used in that series.

γ Average percentage of increase of the various classes; add the percentage increases in each class and divide by the number of classes.

δ Average of the three preceding averages.

The figures with the circle (●) are calculated from incomplete experiments, as one or more classes are missing.

The figures with the letter x are calculated from the figures shown in the various classes in which an average selected in accordance with the experimentally determined figures was substituted for the missing classes. They are in A1, column 4, calculated on a basis of corrected figures.

The series in each column which have the same mark, for instance A1 and B1, represent experiments done under approximately the same conditions and at the same time.

In column R are given the figures for the entire series; in column B are given the figures for the smaller classes (a, c, d, f) and in addition the number of mice (m.) in each class. For instance, 2 m.a. signifies that in a certain experiment there were 2 mice in class a.

We distinguished between the results obtained by means of a combination of both methods and those obtained merely by the first method. The results obtained by means of both methods, and in which the results of both methods corroborate each other, must necessarily be considered more secure than the results based merely on the first method. We shall therefore consider both sets of experiments separately. The experiments carried out by means of both the first and second methods are represented as far as the effects of colloidal copper are concerned in table I. Here is compared the percentage growth from the ninth to the thirteenth day after inoculation (1) of control mice not receiving any injections, (2) of mice injected from the ninth to the thirteenth day with colloidal copper, (3) of mice injected from the second to the sixth and from the ninth to the thirteenth days with colloidal copper, and (4) of mice of the second generation which had been inoculated with pieces of tumor from mice of the first generation which had been injected with colloidal copper from the second to the sixth and again from the ninth to the thirteenth days after tumor inoculation. These mice of the second generation were also injected from the ninth to the thirteenth day. Several experiments were made for the determination of tumor growth in each of these four kinds of mice receiving the treatment outlined. A series of mice served for each experiment (A1, A2, B1, B2, C1, C2, D) in each of the four kinds of animals. Each series was again subdivided into four classes according to the weight of the tumors on the ninth day, and each class again divided into several groups. Each column was again divided into two subcolumns; in subcolumn B is given the percentage growth of the various classes of tumors in each series. The definitions of the various classes according to the weight limits of the various tumors are given in the preceding paper.[6] Such a subdivision is necessary because the percentage growth of tumors within a certain period of time depends upon the size of the tumors at the beginning of that period. At the same time we have to consider that this statement holds good only if we calculate the average percentage growth of a number of tumors. There are quite a number of variations of unknown origin if we consider

[6] Fleisher and Loeb, *Jour. Exper. Med., loc. cit.*

the individual tumors. In subcolumn R is given the percentage growth of the various series of mice. This figure can be computed in various ways. We can compare the weight of all the tumors of a series on the ninth day and on the thirteenth day and determine the percentage increase in the sum of the weights (α). Variations in the number of tumors of various sizes on the ninth day will cause variations in the figures thus obtained, so that the results in different series are not quite comparable. Or we can multiply the average of the percentage increase in weight in each class with the number of tumors in each class, add the figures thus obtained, and divide the sum by the total number of mice used in that series (β). Inasmuch as the number of mice in each class, as far as the valuation of the result of the experimental interference is concerned, is of no importance, it might be of advantage to add the percentage increase in the various classes of each series and divide by the number of classes. This is the best method, if there are enough tumors in each class and if all the classes are represented in each series. This is, however, not usually the case. It will therefore be necessary to interpolate the figures for missing classes by determining the average corresponding to the character of the other classes in that series. If the other classes have a high figure we fill out the vacancy by a correspondingly high figure, determined on the basis of the other experiments, and correspondingly in the case of a low average in the other classes. Thus the error is smaller than if we should altogether omit the figure for one or several classes. In this manner the third figure, γ, was obtained. In the end we took the arithmetical mean of these three figures in each series. This is the fourth figure, δ.

There is a further source of individual deviations from the expected figures in the various series, in each class the percentage increase depending to some extent upon the average class weight on the ninth day, whether it was relatively high or low. If it was relatively high the percentage increase is diminished in conformity with the rule that the larger a tumor is on the ninth day, the less is the percentage increase during the next four days. In table II we give, therefore, the average weight of each class on the ninth day. Each class we divide into two subclasses, one (1) with a relatively

TABLE II.

Class a[7] = 350 mg. and more.
Class c = 225–345 mg.
Class d = 125–225 mg.
Class f = 25–125 mg.

Control (uninjected) mice.		Mice injected with colloidal copper 9th–13th dy.	Mice injected with colloidal copper 2d–5th and 9th–13th dys.		Mice inoculated with tumor from mice previously injected with colloidal copper. Injected 9th–13th dy.	
A		A1 c 286 d 176 f 53			c 247 d 173 f 51	
D a 367 c 240 d 158 f 92	c + 144% d1 + 219% d2 + 208% f1 + 160% f2 + 121%	A2 c 283 d 198 f 68	A2 c 270 d 162 f 87	1 f1 0 5 b2 175% 3 d1 66% 4 d2 136% 4 c1 85% 1 c2 40%		
B1 a 350 c 271 d 180 f 91	1 f1 133% 5 f2 398% 3 d1 175% 3 d2 210% 5 c1 115% 1 c2 138%	B1 a 462 c 281 d 177 f 106	B1 a 466 c 291 d 186 f 92	1 f1 210% 7 f2 165% 5 d1 220% 9 d2 172% 19 c1 197% 6 c2 106%		
B2 a 525 c 291 d 137 f 72	13 f1 460% 5 f2 207% 5 d1 183% d2 4 c1 147% 2 c2 169%	B2 a 446 c 297 d 156 f 92			B2 a 421 c 280 d 160 f 60	9 f1 281% 4 f2 183% 12 d1 261% 6 d2 161% 18 c1 110% 4 c2 86%
C1 a 532 c 276 d 190	1 d1 212% 2 d2 172% 8 c1 141% 4 c2 137%	C1 a 439 c 293 d 162 f 87	C1 a 447 c 291 d 180 f 84	5 f1 345 11 f2 244 8 d1 291 11 d2 244 10 c1 170 6 c2 182		
C2 d 143 f 68	21 f1 321% 14 f2 263% 14 d1 167% 1 d2 113%	C2 a 476 c 284 d 156 f 73			C2 a 447 c 291 d 180 f 84	4 f1 206% 14 f2 310% 18 d1 314% 4 d2 148% 12 c1 151% 11 c2 170%

low and the other (2) with a relatively high class weight. We see that, wherever the number of tumors in both subclasses is suffi-

[7] Each class is divided into two subclasses, the dividing line between these subclasses being in each case the mean between the highest and lowest weight of that class.

ciently high, the lighter tumors have usually a greater percentage growth than the heavier tumors of the same class. Wherever the average weight of a certain class is therefore abnormally low we may expect that the percentage increase is relatively too great as compared with the increase of corresponding classes in other series. We made, therefore, a correction in the figure for class f of the first series in column 4 of table I. Here the average weight of the class is only 51. In this case we deducted about 50 per cent. from the actual percentage increase in weight. The average weight of the tumors on the ninth day in the various series of column 4 is relatively low. It was not essential to make any further corrections. In column 4 of table I we give under γ this corrected figure; in determining δ in this series we also made use of the corrected figure. There exists another factor which is responsible for certain variations in the individual experiments; namely, spontaneous retrogressions which may take place in class f. The number of spontaneous retrogressions in class f may vary; and this accounts for the relatively low average for class f in experiment D. In this experiment twenty-four tumors grew distinctly and twelve grew either very much below the average, or did not grow at all. This is the only experiment where this factor is of any significance.

On the whole, the figures designated γ in table I seem to be best for comparison of the percentage increases in weight in the different series of experiments.

A study of the averages of the various series as given in the summary in table I shows: (1) That the average percentage increases determined according to the different methods used by us give similar results; the greatest variation is found in column 4. (2) We find that four injections of colloidal copper retard the percentage growth of the tumors noticeably. If we adopt for comparison the figures γ and δ, uninjected control tumors grow 174 per cent. (180 per cent.), while tumors of mice injected with copper grow 110 per cent. (107 per cent.). (3) However, if we immunize mice by giving them four preliminary injections of colloidal copper from the second to the sixth day the inhibiting effect of the colloidal copper disappears, perhaps not altogether, but it certainly becomes insignificant; the respective figures for the immunized mice are now

167 per cent. (165 per cent.), which are only slightly below those of the control mice. (4) The fourth column shows a still more decided immunity of tumors of the second generation which had been subjected to injections of colloidal copper in the former generation of mice. Here the corresponding figures are 189 and 206 per cent. respectively. These figures are even slightly higher than those of uninjected control mice. Whether this increase is due to the variable factors mentioned previously or to a slight stimulating effect of injections of colloidal copper in the second generation, we are not ready to decide, although we are inclined to accept, at least at present, the first interpretation. (5) If we compare in the summary the figures for the percentage increase of the individual classes, we find that copper is about equally active in all four classes and that the immunization also affects all four classes. (6) If we compare instead of the figures given in the summary the individual series included in the summary we see that the figures in the various series are consistent with those of the summary, although there exist individual variations, especially in series in which the number of mice used is relatively small. It is necessary in valuing the relative importance of the figures in the various series to consider the number of mice used in a series; the series with a larger number of mice show on the whole a much smaller deviation from the average than series with a smaller number of mice. It is furthermore advisable to compare series in the same experiment. The conditions in different experiments vary more than in the same experiment, the kind of mice used and the tumor used for transplantation being more similar in the same experiment than in other experiments.

Table III shows the results obtained by the first method, in which we estimated the growth of the tumors by palpation. Table I gives the results obtained by weighing in the same experiments. We see that on the whole the results obtained by both methods agree very well, although in a few classes the results do not agree completely. Table IV presents the results in different classes (a) of uninjected controls, (b) of animals injected with copper without and (c) with previous immunization. The results were obtained with the aid of the first method. We see again that all classes are about equally

TABLE III.

Normal (uninjected) controls.	Mice injected with colloidal copper from 9th–13th dy.	Mice injected with colloidal copper from 2d–5th and 9th–13th dys.	Mice inoculated with tumor from mice previously injected with colloidal copper. Injected from 9th–13th dy.
H a 2 +[8] c 9 + 1 − d 16 + 2 − f 22 + 1 −	A1 c 2 + 3 − d 7 + 2 − f 3 + 7 −		A1 c 15 + 3 − d 12 + f 10 + 2 −
D a 4 + c 8 + d 31 + 3 ? 1 − f 24 + 7 + 5 −	A2 c 3 + 3 − d 2 + 2 − f 9 + 14 −	A2 c 5 + d 6 + 1 − f 5 + 1 −	
B1 a 1 + c 6 + d 5 + 1 − f 5 + 1 −	B1 a 8 + 11 − c 11 + 19 − d 4 + 4 − f 3 + 1 −	B1 a 11 + 1 − c 18 + 3 − 5 d 11 + 2 − f 7 + 1 −	
B2 a 10 + c 6 + d 4 + 1 − f 15 + 3 −	B2 a 4 + 6 − c 7 + 7 − d 5 + 7 − f 7 + 6 −		B2 a 7 + 1 − c 17 + 5 − d 17 + 1 − f 10 + 3 −
C1 a 3 + c 11 + 1 − d 3 +	C1 a 7 + 7 − c 7 + 8 − d 7 + 7 − f 8 + 7 −	C1 a 7 + c 16 + d 18 + 1 − f 12 + 4 −	
C2 d 11 + 4 − f 29 + 6 −	C2 a 4 + 4 − c 11 + 9 − d 7 + 8 − f 11 + 12 −		C2 a 6 + c 18 + 4 − d 20 + 2 − f 17 + 1 −

affected as far as the retardation in growth through colloidal copper as well as the processes of immunization are concerned. We carried out one similar experiment with hirudin. The various samples of hirudin vary somewhat in their efficacy and the one at our disposal at that time was rather weak. We have further to consider that in the case of the hirudin the results are complicated by hemorrhages into the tumor.

[8] In tables III and IV, + = grew; — = did not grow; ? = questionable.

TABLE IV.

Growth and Non-Growth as Shown by Palpation.

Normal control.	Immunized.	Colloidal copper controls.
a 14 +	a 32 + 2 —	a 23 + 29 —
c 25 + 1 —	c 90 + 15 — 5 ?	c 36 + 39 —
d 24 + 8 —	d 84 + 8 —	d 34 + 29 —
f 48 + 11 —	f 70 + 12 —	f 41 + 47 —

In this experiment fifty-three mice were kept as controls, without injections; sixty-eight mice received injections from the ninth to the thirteenth day, gradually increasing doses of hirudin being given, and sixty-nine mice received four injections from the second to the sixth day and then again from the ninth to the thirteenth day. On examining the mice on the thirteenth day we found many hemorrhagic tumors in the second lot injected only from the ninth to the thirteenth day. The hemorrhages were very much less in the third lot which had received preliminary injections from the second to the sixth day. The increase in weight was much more considerable

TABLE V.

Normal control mice.		Hirudin injected 9th–13th dy.		Hirudin injected 2d–6th and 9th–13th dys.	
53 mice.		68 mice.		69 mice.	
H 174% 194% 166% 178%	a + 113% c + 143% d + 147% f + 259%	139% 150% 147% 145%	c + 126% d + 150% f + 166%	220% 203% 207% 211%	c + 208% d + 237% f + 177%

in the third than in the second lot. The preliminary injections seem, therefore, to have exerted an immunizing effect. We are here confronted with the fact that while the tumors of mice receiving hirudin injections from the ninth to the thirteenth day grew less than the uninjected control mice, the mice of the third lot grew

TABLE VI.

	Injected 2d–6th dy. with:	Injected 9th–13th dy. with:		Controls receiving injections of colloidal copper or hirudin 9th–14th dy. only.	
A Injected 2d–6th and 9th–13th dys.					
	Copper	Copper 36 R	244 G[9] (87%)	131 R[9]	120 G (47%)
	Copper	Hirudin 28 R	9 G (24%)	16 R	3 G (16%)
	Hirudin	Hirudin 38 R	183 G (83%)	94 R	63 G (40%)
	Hirudin	Copper 28 R	30 G (52%)	23 R	27 G (54%)
B Injected before inoculation					
	Injected before inoculation with:	Injected 9th–13th dy. with:			
	Copper	Copper 3 R	53 G (95%)	33 R	41 G (56%)
	Hirudin	Hirudin 58 R	38 G (40%)	45 R	21 G (32%)
C Transplanted tumor injected after the preceding generation had been injected					
	Preceding generation injected with colloidal copper Copper series				
	Copper injected 9th–13th dy.	62 R	441 G (87%)	183 R	122 G (40%)
	Hirudin injected9th–13th dy.	57 R	24 G (30%)	39 R	9 G (19%)
	Preceding generation injected with hirudin Hirudin series				
	Hirudin injected 9th–13th dy.	61 R	83 G (58%)	66 R	22 G (25%)
	Copper injected 9th–13th dy.	62 R	36 G (37%)	51 R	30 G (37%)
D Injected 9th–13th dy.					
	Copper	371 R	277 G (43%)		
	Hirudin	184 R	102 G (36%)		
	38% of these retrogressed Of these 38% about 80% resumed growth when injections were stopped after the 4th dy., and about 70% resumed growth if injections were continued for 6 or 7 dys.				

[9] G = tumors growing at the normal rate; R = tumors with retarded growth or no growth.

more than the control mice. This is probably due to the fact that the weight of the tumors increases after injections of hirudin on account of edema and hemorrhages into the tumor; the average of the percentage increase of the tumors in the hirudin series is therefore less than the figures indicate. We must, however, at the present time admit that possibly some accidental factors may in addition be of importance (table V).

We carried out further experiments in order to determine the specificity of the immunity conferred by a first set of injections. We wished to determine whether or not preliminary injections of hirudin weaken the action of later injections of colloidal copper, and *vice versa;* and also whether injections of hirudin or colloidal copper given before inoculation with the tumor have the same weakening action on the effect of later injections of hirudin or colloidal copper given nine to thirteen days after inoculation. If that should be the case the immunizing action would take place through some organ or tissue of the injected mouse, independently of the tumor cells. For these experiments which were made at an earlier stage of our work we used the first method of determining the growth exclusively. We added, however, wherever it was feasible the figures obtained in later experiments in which we used for other purposes both the first and second method combined. Table VI shows the results of these experiments. Part D shows that more than one half of the tumors injected with either hirudin or colloidal copper are more or less retarded in their growth as a result of the injections. There does not seem to be a marked difference between the action of colloidal copper and of hirudin. Part B shows that injections of colloidal copper given before inoculation of the tumor lower markedly the effect of subsequent injections of colloidal copper. In the case of hirudin this lowering effect was apparently present only to a very slight extent. Part A shows that injections of colloidal copper given from the second to the sixth day weaken the effect of injections given from the ninth to the thirteenth day and that the same holds good in the case of hirudin, but that a first set of injections of hirudin does not markedly influence a second set of injections of colloidal copper, and that a first set of injections of colloidal copper does not influence markedly a second

set of injections of hirudin. Part C shows that a first set of injections of colloidal copper or hirudin given in one generation of tumor mice from the second to the sixth and from the ninth to the thirteenth days weakens the effect of a later set of injections given in the following generation of tumor mice, and again that this effect is specific, colloidal copper immunizing the tumor cells only against colloidal copper, and hirudin only against hirudin. In the case of hirudin the immunizing effect is apparently not quite as marked as in the case of colloidal copper.

In appraising the value of the results obtained we must be aware that in the best case only the principal facts can be established with the aid of the first method. Slight quantitative differences have to be disregarded altogether. As far as experiments have been carried out by both methods the results agree. We may therefore conclude that experiments carried out so far indicate that the immunization produced through preliminary injections of hirudin or colloidal copper is a specific one, each protecting only against later injections with the same substance. And we may also conclude that injections of colloidal copper, and probably also hirudin, given before the inoculation with the tumor have also an immunizing effect against later injections. Immunity against the action of colloidal copper and hirudin depends therefore on an immunity acquired by the host organism as well as on an immunity acquired by the tumor cells themselves.

In the case of hirudin we have also other evidence that processes of immunization take place in the organism of the injected mice; five to six intravenous injections of hirudin given on consecutive days immunize the animals against the anticoagulating effect of hirudin.[10] This effect can be demonstrated *in vitro* as well as *in vivo*.

We have still to consider another possible explanation for the results which we obtained; namely, the possibility that a selection of tumors naturally immune takes place, rather than active immunization of the tumors. This interpretation cannot be accepted (1) because the tumors subjected to the influence of the substances and the control tumors took their origin from the same tumor a few

[10] Vera, M., and Loeb, L., *Jour. Biol. Chem.*, 1914, xvii, p. xxv.

generations back. It can further be excluded (2) because after injection with colloidal copper from the second to the sixth day the majority of tumors grew under the influence of injections of colloidal copper given from the ninth to the thirteenth day. An accidental selection can therefore not very well take place under these conditions, because the chance that naturally susceptible tumors are used for transplantation is as great as the selection of naturally immune tumors. (3) If we compare the influence of colloidal copper on tumors that developed after transplantation from tumors whose growth had been retarded after injections of colloidal copper, and on those that developed after transplantation of tumors whose growth had not been influenced by colloidal copper, we find that both series are equally immune against the action of colloidal copper.

Tumors that had been transplanted from tumors that had proven resistant to the influence of colloidal copper gave the following result: 31 grew normally and 3 were retarded in their growth under the influence of colloidal copper. Tumors that had been transplanted from tumors that had been retarded in their growth under the influence of colloidal copper gave this result: 38 grew normally and 3 were retarded in their growth. Tumors derived from tumors that had grown well under the influence of colloidal copper gave the following result after injection of hirudin: 10 were retarded and 4 grew well. Tumors derived from tumors that had been retarded in their growth under the influence of colloidal copper gave the following result: 10 were retarded and 8 grew well under the influence of hirudin. We may therefore conclude that the results we obtained are due to an immunization of the tumor cells and not to a selection of naturally resistant tumors.

Our results point to the conclusion that the acquired immunity is a specific one. If this is the case, we should be able to affect, through injection of hirudin, tumors that had been resistant to the action of colloidal copper. This we found to be the case. Tumors of mice that had been injected with colloidal copper from the ninth to the thirteenth day and had grown normally under the influence of this substance, were injected with hirudin from the thirteenth to the sixteenth day. Of 36 animals thus treated, the tumors

retrogressed in 6, were retarded in their growth in 19, and grew normally in 11 animals. Hirudin had therefore its usual effect on tumors after colloidal copper had previously failed to exert any influence on the same tumors.

We also had to consider the possibility that the immunization was due to the fact that after injections from the second to the sixth day after inoculation the general health of the tumor mice was less affected through subsequent injections from the ninth to the thirteenth day, and that in consequence of the better health of the mice the tumors grew better in animals having had the first set of injections. However, we found that the appearance of both sets of animals, those having received two sets of injections and those having received only one set, were equal. In a certain number of animals we determined the effect of the injections on the weight of the animals and obtained the following results:

After inoculation	Average weight of mice receiving colloidal copper 9th–13th dy.	Average weight of mice receiving colloidal copper 2d–6th and 9th–13th dys.
2 dys.	16.2 gm. (100 mice)	16.7 gm. (100 mice)
9 dys.	16.1 gm. (78 mice)	16.2 gm. (75 mice)
13 dys.	16.2 gm. (63 mice)	16.2 gm. (66 mice)

We see that there is very little difference in weight on the ninth and on the thirteenth day in the mice of the two series. During the first injections, from the second to the sixth day, the mice of the second series lost slightly in weight, 0.5 of a gram on the average. In this series the weight remained constant during the second set of injections, while it increased slightly (0.1 of a gram) in the series not receiving the first set of injections. We may therefore conclude that general conditions of health cannot account for the immunity that we noticed.

We saw that as a result of the presence of several variable factors the growth differs considerably in individual tumors. This necessitated the use of large numbers of tumors, in order to test the effect of various factors on tumor growth. We are conscious of the fact that results based on statistical methods do not possess the same degree of certainty as results of experiments in which individual variations do not occur. On the other hand, the number

of tumors observed by us is unusually large. We lay at the present time the main emphasis on the publication of the actual results obtained in this as well as in the preceding communication, without considering the conclusions as yet as more than probable. However, we hope that the opportunity will soon present itself to us to carry out another series of similar experiments, and we shall then state whether or not the further experiments confirmed our previous work. With this understanding, implying the restrictions mentioned, we may point out some of the further conclusions that can be drawn from our work.

1. Our work shows that through injections of solutions of colloidal metals changes take place in the body which tend to counteract the further efficacy of the colloidal metal. It is probable that as the result of these reactions the colloidal solution itself within the body is changed to such an extent that it is less efficacious.

2. If, as our experiments seem to indicate, the immunity is in part localized in the tumor cells and if this acquired immunity is transferred to successive cell generations, and especially if this immunity is a specific one, it is probable that the various substances that we use attack the tumor cells directly and act only indirectly through changes in the circulation or in the blood vessels of the tumor or of its environment. The latter action may, however, exist side by side with the direct one on the cells. In a former publication[11] we have already drawn attention to this possibility.

3. Such processes of immunity as those that we observed would be an important factor in the practical application of chemotherapy. They would tend gradually to diminish the efficacy of the substances we use, provided that we do not apply substances causing a rapid destruction of the tumor. On the other hand, the specificity in these processes of immunization would make it possible to counteract the injurious influence of immunization established against one substance, by using new substances after the usefulness of the first substance has been noticeably diminished.

[11] Loeb and Fleisher, *Jour. Am. Med. Assn., loc. cit.*

SUMMARY.

1. Through a set of preliminary injections of colloidal copper or hirudin, given from the second to the sixth day after transplantation, we can weaken the effect of injections of the same substances given from the ninth to the thirteenth day after transplantation.

2. Injections of colloidal copper given before the transplantation of the tumor lead to a weakening of the effect of injections given from the ninth to the thirteenth day after transplantation.

3. The same result can be obtained if we inject colloidal copper or hirudin into mice from the second to the sixth and from the ninth to the thirteenth day after transplantation, and use these tumors at the end of the series of injections for further transplantation into another set of mice; the developing tumors are more resistant to the action of colloidal copper or hirudin than new control tumors.

4. We may therefore conclude that the immunity has two sources: (a) it is based on changes taking place somewhere in the host organism; and (b) it is localized in the tumor cells themselves which transfer this immunity to the following generations of tumor cells.

5. The immunity acquired against colloidal copper does not protect noticeably against the action of hirudin, and *vice versa*. The immunity is therefore specific.

6. We discuss certain general conclusions which may be drawn from these experiments. We expect to test the validity of these results in further experiments.

DO SUBSTANCES INHIBITING TUMOR GROWTH EXERT A RETARDING INFLUENCE ON THE REGENERATION OF THE SKIN?*

By WILLIAM E. LEIGHTON, M.D.

(*From the Department of Pathology of the Barnard Free Skin and Cancer Hospital, St. Louis.*)

In a series of studies carried out in this laboratory[1] the effect of various substances on tumor growth has been determined. Several substances, especially colloidal copper, hirudin, nucleoprotein, and casein were found to be active.

In order to contribute to the understanding of the mechanism of these substances, it was considered of interest to study their influence on other processes besides tumor growth, and at Dr. Loeb's suggestion I studied the effect of these substances on wound healing in the mouse.

The problem that I undertook was to determine if possible whether any change, macroscopic or microscopic, could be produced through the intravenous injection of these substances in a wound in the process of healing.

The technique adopted consisted in excising in each experiment a small piece of skin from the back of each of eight mice, care being taken to make excisions of about the same size. At first the wounds were protected by a colloidin dressing, but later this was omitted as the wounds seemed to heal better without this covering; and when the mice were kept in separate boxes no noticeable infection followed and the wound healed normally.

On the third day after the wound was made, four of the mice were injected through the vein of the tail with the substance to be tested, and injections were repeated on four successive days. The mice were killed on the eighth day and the wound with the surrounding skin was excised for microscopic study.

* Received for publication, July 16, 1914.

[1] Fleisher, M. S., and Loeb, L., *Jour. Exper. Med.*, 1914, xx, 503.

The four control mice were either not injected at all or were injected intravenously with normal salt solution or distilled water. They were also killed on the eighth day and the wound with its surrounding skin was excised for microscopic study.

When a mouse died or was killed by the injection at an earlier date, the wound was excised and at the same time a control animal was killed and its wound excised for study.

Altogether twelve experiments were carried out with the four substances active in the case of mouse tumors. The wounds which were excised on the eighth day showed no difference macroscopically. Microscopically it was found that the regeneration of the epithelium, as well as of the connective tissue and blood vessels, was approximately the same in control mice, and in the mice injected with the various substances. We may therefore conclude that none of the substances mentioned has a noticeable influence on wound healing.

CONCLUSIONS.

Repeated injections of colloidal copper, hirudin, nucleoprotein, and casein, which have a definite retarding influence on tumor growth, given to mice during the process of wound healing do not produce any noticeable influence on the course of regeneration.

THE EFFECT OF GENTIAN VIOLET ON PROTOZOA AND ON TISSUES GROWING IN VITRO, WITH ESPECIAL REFERENCE TO THE NUCLEUS.*

By D. G. RUSSELL, M.D.

(*From the Laboratory of Surgery of Yale University, New Haven.*)

PLATE 26.

In the original communication of Churchman[1] on the selective bactericidal action of gentian violet, published two years ago, the suggestion was made that similar studies should be carried out on the effect of the dye on growing tissue. This communication contains the report[2] of work undertaken in that direction. The work was primarily undertaken to settle two questions raised by the observations on the effect of gentian violet on bacteria. These two questions were (a) whether the nucleus of a single-celled organism could be stained during life, and (b) whether such a stained organism was capable of further reproduction.

It was noticed early in the gentian violet studies that motile organisms not killed by the stain (violet-negative organisms) retained their motility even though deeply stained; and that these stained violet-negative organisms when transplanted to agar slants grew equally well with the control smears of the unstained bacteria. The retention of motility by the stained organisms might be explained as a survival phenomenon; and the growth of the transplants made from the stained specimens might be regarded as arising, not from the organisms in the smear which had taken the stain but from the few in the smear which had escaped it. It

* The experimental work on which this communication is based was presented by the author in a graduation thesis required for the degree of Doctor of Medicine in Yale University. Received for publication, July 28, 1914.

[1] Churchman, J. W., *Jour. Exper. Med.*, 1912, xvi, 221.

[2] Churchman, J. W., and Russell, D. G., *Proc. Soc. Exper. Biol. and Med.*, 1913–14, xi, 120. This was the preliminary report of this work.

seemed altogether likely, from other observations, that this explanation was not the correct one, and that the violet-negative organisms actually took the stain during life. Still, definite proof was lacking that gentian violet in these experiments was acting as a true vital stain. To furnish this proof and to investigate the further problem (raised but not solved by the experiments with bacteria) as to whether the violet dye stained the nucleus of the protoplasm, experiments have been done with protozoa, adult living tissue, and embryonic living tissue.

THE EFFECT OF GENTIAN VIOLET ON PROTOZOA.

The organisms first used for this purpose were paramecia from a pedigreed race furnished by Professor Woodruff. The effect of the dye was investigated in two ways. In the first experiments the organism was stained and then transplanted. In the second series the organism was grown in media containing the dye.

TECHNIQUE.

In the first series of experiments the following technique was used: A single paramecium was placed in a watch-glass containing stain of varying dilutions (1 to 200, 1 to 10,000, and 1 to 100,000) and stained for half a minute, one minute, and five minutes, respectively. The organism was then recovered with a fine pipette, washed thoroughly in two or three washings of distilled sterile water, transplanted to a medium of 0.025 per cent. beef broth (Woodruff and Baitsell,[3] 1911) which had been inoculated with *Bacillus prodigiosus*. The paramecia were studied in drops of this medium on depression slides, which were kept in large covered glass dishes with water in the bottom to prevent evaporation.

In these experiments the organism was found to stain deeply in all the gentian violet dilutions used. The rapidity with which the organisms took up the stain and lost their motility was in direct ratio to the concentration of the dye. In the strongest solution the organism became promptly stained and motility was rapidly lost. In the weaker solutions the paramecia swam about actively

[3] Woodruff, L. L., and Baitsell, G. A., *Jour. Exper. Zoöl.*, 1911, xi, 135.

and normally and it was quite easy to watch the absorption of the dye by both cytoplasm and nucleus. This staining of cytoplasm and nucleus took place while motility was still retained and while the cilia were still whipping. The dye, however, soon caused a retardation of motility and later a cessation; and then the organisms became so deeply stained that no structure could be made out. Such an organism when transplanted did not resume its activity and in a short time broke up into unrecognizable debris.

In the second series of experiments the effect of the dye was studied by transplanting the paramecia into media containing gentian violet.

The technique was as follows: A single paramecium was planted on a depression slide in a drop of 0.025 per cent. beef broth, to which gentian violet in varying strengths (1 to 400 up to 1 to 10,000,000) was added. These drops had been previously inoculated with *Bacillus prodigiosus*. Evaporation was prevented as before.

The effect of the dye in these experiments was sharp and constant. In dilutions stronger than 1 to 500,000 the nucleus as well as the cytoplasm soon became distinctly stained and the cell outline sharply defined. This took place while active motility, both rotatory and progressive, was still retained, and while the cilia were still whipping violently. In a short time, however, the motility diminished and the organism finally came to a standstill. The cilia continued for some time to wave but later these also stopped. The organism then gradually swelled until the cell membrane ruptured, allowing the protoplasm to escape; and the organism either appeared as a deeply stained motionless and structureless mass, or else persisted only as unrecognizable debris. In strengths of 1 to 100,000 cessation of motility occurred in a few minutes. In 1 to 500,000 dilutions some of the organisms were still motile forty-eight hours after immersion but none of them survived after three days and none divided. In 1 to 1,000,000 dilutions fission was always delayed and in a number of cases prevented, though some organisms reproduced slowly and a few were apparently faintly stained in the dividing stage. In strengths weaker than 1 to 1,000,000 there were suggestions of staining in a few instances, but no apparent inhibition of motility or reproduction.

A number of similar observations were made on oxytricha. The technique was similar to that used with paramecia. The results with this organism, though in general the same as those just recorded for paramecia, were not quite so well defined and constant, owing to the fact that this organism is more difficult to handle without injury.

There was no question that the nucleus in these experiments with paramecium and oxytricha was deeply stained, while both organism and cilia were actively motile; and if motility be regarded as a certain indication of life the observations warrant the conclusion that gentian violet is a true vital nuclear stain. We were unable, however, to observe cell division in a definitely stained organism; and the motility in these experiments might well be interpreted as a survival phenomenon in dead or dying organisms. The nature of the experiments was therefore changed and the effect of the dye on growing animal tissue was investigated.

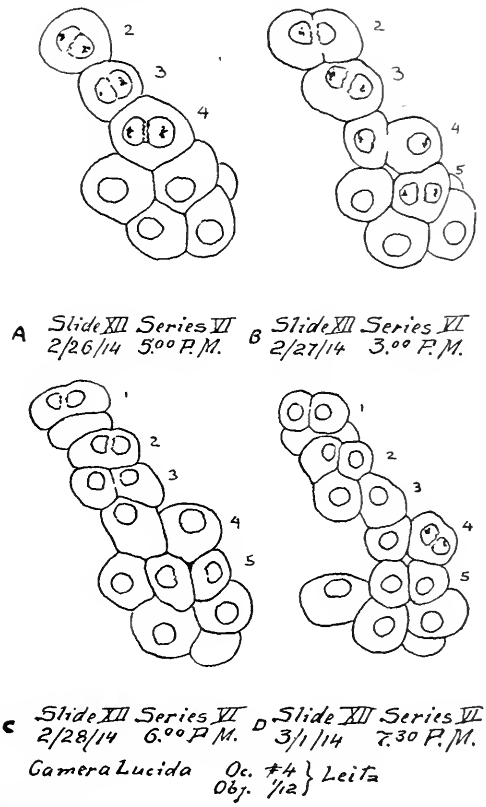
THE EFFECT OF GENTIAN VIOLET ON ADULT FROG TISSUE GROWING IN VITRO.

In the first series of experiments connective tissue from the adult frog was grown in frog plasma, according to the technique described by Harrison.[4] The effect of the gentian violet was studied by adding it to the plasma into which the tissue plants were made. The dye had no effect on the clotting of the plasma, as was to be expected from the observations of Churchman on the effects of gentian violet on enzymes.[5] The connective tissue was found to grow rather poorly, particularly in the early experiments, in which very strong dilutions of stain were used. In preparing a series of connective tissue plants from the fascia of the upper abdominal muscles, pericardium was accidentally included; and the endothelial cells in these transplants showed definite growth, while the connective tissue cells were apparently inhibited by the dye. In the first experiments the plasma contained gentian violet in a dilution of 1 to 2,000. Definite endothelial growth occurred even in this strength but in all subsequent experi-

[4] Harrison, R. G., *Anat. Rec.*, 1906–08, i, 209; *Jour. Exper. Zoöl.*, 1910, ix, 787.

[5] Churchman, *Proc. Soc. Exper. Biol. and Med.*, 1913–14, xi, 54.

ments a 1 to 20,000 dilution was used. Here the growth of the endothelium was active, keeping pace with the controls. In some cases, indeed, the growth in the stained media seemed to outstrip that in the controls. This growth occurred in spite of the fact that the tissue plant, soon after being placed in the dye-containing plasma, became deeply stained. That this tissue plant was stained while alive and growing was demonstrated by transplanting a piece of the tissue which had grown in the dye-containing plasma to the unstained plasma, where the growth of the deeply stained ex-plant continued. These observations made it clear that the cell nucleus



TEXT-FIG. 1. Diagrams made on four succeeding days of endothelial cells of the pericardium of an adult frog, grown in plasma containing gentian violet 1 : 20,000, showing that cell division was observed from beginning to completion. Figures (1, 2, 3, 4, and 5) have been placed so that it is possible to follow the individual cells in the four diagrams.

itself was stained and that this staining was vital and did not interfere with growth. The staining of the plasma and nucleus is well shown in figure 1.

In a number of specimens it was possible to follow the division of these stained nuclei and to observe the whole process with the oil immersion lens (text-figure 1). In these early experiments with adult tissue, however, clear karyokinetic figures were not seen. In one specimen a piece of peritoneum from the female frog was planted in stained plasma. The cilia were observed to remain actively motile for a week, and active growth of the transplant produced new ciliated endothelium. In another experiment the tissue was kept alive and growing for over two weeks in the stained plasma by transplanting the tissue every three or four days into freshly stained plasma.

In no experiment on the growth of living tissue in stained plasma did an infection ever occur, while in the controls infection with bacteria was not infrequent. We were also struck by the fact that endothelial cells grew much more readily in the presence of the dye than connective tissue cells, and we are inclined to the view that we are dealing here with a selective action not unlike that exercised by this dye on bacteria.

Another fact brought out by these experiments was the power of the growing animal cells to change the dye so that its color disappears. That a similar change takes place in the animal body was pointed out by Churchman and Herz in a previous communication.[6] It was there shown that large amounts of gentian violet injected into the ear vein of the rabbit soon disappeared from the blood; and that the mucosa of the tongue and lips, though at first deeply stained, in a short time (about forty-eight hours) lost their violet color. Animals killed a few days after the injection showed no trace of the dye, nor did it appear as such in the urine.

THE EFFECT OF GENTIAN VIOLET ON EMBRYONIC FROG TISSUE GROWING IN VITRO.

In this series of experiments, tadpoles of *Rana silvatica* and *Rana pipiens* in the pre-gill and gill stages were used. The effect of

[6] Churchman, J. W., and Herz, L. F., *Jour. Exper. Med.*, 1913, xviii, 579.

gentian violet was studied in preparations of the tissues planted in stained plasma prepared according to the following technique.

A tadpole was washed thoroughly in sterile distilled water and then placed in sterile 0.3 per cent. saline solution. Under a Zeiss binocular a small incision was made in the skin and the flap turned back. In *Rana silvatica* the skin could not be separated from the underlying connective tissue there, so that cultures made from pieces of the skin of the flap contained both epithelium and mesenchyme. In *Rana pipiens* the skin could be easily separated from the connective tissue and so pure cultures of epithelium and mesenchyme were made. In tadpoles in the gill stage the tissue cultures were made from pieces of the gills (which could easily be cut off), from the tail (where the pigment is practically absent), and from the heart. The tissue plants were all made into plasma containing stain of varying dilutions (1 to 4,000 up to 1 to 20,000); and controls were simultaneously made in unstained plasma.

The embryonic tissues remained alive and grew in the presence of the stain. In the mixed cultures of epithelium and mesenchyme the epithelial cells grew rapidly, while the mesenchyme was apparently retarded by the action of the dye. The same selective action of the stain was also brought out in the pure cultures of epithelium and mesenchyme. In the former, growth was active and rapid, while in the latter growth was slight and in some cases entirely prevented. In the preparations made from the gills, both the epithelial and endothelial cells grew well in the stained plasma; while in the preparations from the heart pure cultures of endothelial cells were obtained.

In one series of epithelial tissue cultures, ciliated epithelium was present and the cilia remained actively motile for over two weeks in the presence of gentian violet in the dilution of 1 to 20,000. The growth of epithelium in stained plasma (dilution of 1 to 4,000) was not as active and rapid as in the weaker dilutions used. In the weaker dilutions growth of epithelium and endothelium was active and the nuclei were slightly stained. These stained nuclei did not stand out as clearly and distinctly as those in the more slowly growing adult endothelium but were definitely violet in color. In a small series of experiments, made very carefully

with this point in view, we have observed the division of the definitely stained nuclei and the presence of karyokinetic figures in these dividing stained nuclei (figure 1).

In the experiments with embryonic tissue the effect of the growing cells on the stain was much more marked than in the case of growing adult tissue. In twenty-four hours the color of the stain was decreased very markedly and in dilutions of 1 to 20,000 the violet color was almost entirely removed by the end of four days.

These experiments prove that gentian violet penetrates by diffusion the living cell and its living nucleus; for the staining of animal cells which subsequently grow in a normal manner cannot be interpreted as a survival phenomenon, and the observation of the staining of the karyokinetic figures in these growing cells demonstrates that the dye penetrates into the nucleus itself.

CONCLUSIONS.

1. Gentian violet may be regarded as a true vital nuclear stain.

2. Embryonic and adult tissue of the frog will grow *in vitro* in the presence of gentian violet of a far stronger dilution than that necessary to kill many bacteria. In these experiments, for example, successful tissue growths were obtained when gentian violet 1 to 20,000 was used, yet *Bacillus subtilis* will not grow in 1 to 100,000 dilution and grows very badly in 1 to 1,000,000 dilution. This fact may simplify the technique of the growth of certain tissues by eliminating the risk of bacterial contamination.

3. The use of stains in the plasma in which tissue is grown will probably facilitate the study of nuclear growth.

4. Gentian violet appears to have a certain selective action on tissue similar to that exercised by the dye on bacteria. Certain observations made last year in this laboratory (too few to serve as more than a suggestion) seem to indicate that another dye (methylene blue) acted as a stimulant to the growth of connective tissue. These leads should be followed out and the effect of various stains studied in the hope of discovering dyes which will exercise a sharp selective action on growing tissue.

5. The growth of animal cells in a strength of dye much more than sufficient to kill many pathogenic organisms lends encourage-

ment to efforts now being made in this laboratory to apply the observations on the bactericidal effect of gentian violet and allied stains to the treatment of disease. Moreover, the ability of growing cells to rid themselves of this dye would indicate that it may be possible to apply the dye to infected tissue and count on the cells to eliminate the material after its work had been done.

EXPLANATION OF PLATE 26.

FIG. 1. New growth of epithelial cells, from the skin of the tail of *Rana pipiens* in the gill stage, growing in stain-containing plasma (gentian violet 1:4,000). The nuclei are distinctly stained, showing dividing forms with suggestion of staining of the chromosomes. The cell outlines are very distinct and the cytoplasm is stained. Drawn in natural colors with the camera lucida, ocular No. 4, objective $\frac{1}{6}$, Leitz.

THE EFFECT OF VARIOUS TISSUE EXTRACTS UPON THE GROWTH OF ADULT MAMMALIAN CELLS IN VITRO.*

By ALBERT J. WALTON, M.S.

(*From the Bacteriological Laboratory of the London Hospital, London.*)

PLATES 27 TO 31.

It was shown in a previous communication[1] that the extent of growth of adult mammalian tissue is to a large extent dependent upon the nature of the plasmatic medium used. It then became necessary to investigate the effects of various tissue extracts upon the growth, for it is possible that the variations in the plasmata of different animals might in part be dependent upon the secretions of various glands. There is already considerable clinical evidence that glandular secretions affect the growth of various tissues *in vivo*. Carrel has also shown[2] that the growth of tissue *in vitro* is stimulated to a marked degree by certain tissue extracts, especially, in the case of the chick, by the extract of chick embryo, but the presence or absence of any specific action of the extracts on different tissues has not been investigated. This communication will deal with the effects of various extracts of adult tissue upon the growth of cells obtained from adult mammals.

TECHNIQUE.

In all cases the plasma and tissues of adult rabbits were used both for cultural purposes and for preparing the extracts. The cultures were prepared by Carrel's technique in the manner I have previously described,[3] but after the piece of tissue had been placed in the

* The expenses connected with this work were defrayed by a grant from the London Hospital Research Fund. Received for publication, August 17, 1914.

[1] Walton, A. J., *Proc. Roy. Soc. London*, 1914, series B, lxxxvii, 452.

[2] Carrel, A., and Burrows, M. T., *Jour. Exper. Med.*, 1911, xiv, 244. Carrel, A., *idem*, 1912, xv, 516; 1913, xvii, 14; 1913, xviii, 287.

[3] Walton, A. J., *Jour. Path. and Bacteriol.*, 1914, xviii, 319.

plasma on the sterile cover-slip, a little of the fluid extract, equal in quantity to about one half the amount of plasma, was added with a sterile pipette and the fluids were mixed with a cataract knife. Almost immediately after the addition of the tissue extract the plasma coagulated. The preparation was then inverted over the cell slide and sealed in the usual way. In all cases, both in the primary and in the subcultures, an equal number of controls were made, the tissue in this case being grown in simple plasma. This was essential in every case for, as I have previously shown, the amount of growth varies considerably with the use of different specimens of plasma. It was also necessary to have controls in which the plasma was mixed with some inert fluid so that the changes in growth brought about by dilution should not be confounded with those caused by the addition of the extract. For this purpose a certain number of preparations were made in which the plasma was mixed with about one half of its volume of Ringer's fluid. Under these conditions it was found, as Carrel has previously shown,[4] that growth was somewhat accelerated. Allowance was made for this acceleration in deducing the results of the experiments.

Preparation of the Extracts.—The extracts were prepared by cutting or grinding up the given tissue in sterile Ringer's fluid, leaving the mixture to stand for a short while, and then centrifugalizing it. The supernatant fluid was then pipetted off and used as the extract. The fine division of the tissue in a sterile condition presented some difficulties. In the case of soft tissues such as the testicle, liver, spleen, and kidneys these difficulties were overcome by the use of the following instrument.[5] A brass syringe of a capacity of about seven cubic centimeters was made, the piston of which had two cross bars projecting from its face. These cross bars were made to sink in flush with the rest of the face of the piston, but were kept projecting by means of springs. When so projecting they acted as cutters, but when the piston was pressed down they could sink into it and the face of the piston was then smooth. The nozzle of the syringe was made to unscrew and between it and the barrel was inserted a perforated steel plate. The perforations were ta-

[4] Carrel, A., and Burrows, M. T., *Jour. Exper. Med.*, 1911, xiii, 562.

[5] I am indebted to Mr. H. S. Souttar for assistance in devising this instrument.

pered so that the narrow openings were nearest the piston and the edges on this surface were therefore sharp. The syringe was sterilized and a piece of the required tissue inserted into it. On rotating the piston the cross bars forced the tissue round so that it was cut on the perforations in the steel plate. The piston being at the same time slowly pushed home, the cut fragments of the tissue were forced through the plate. The cross bars sinking in as the piston was forced home, no dead space was left and all the tissue was thus used up. A small amount of Ringer's fluid was now sucked up into the syringe and formed an emulsion with the cut tissue. The mixture was then ejected into a sterile glass tube, corked, and, after standing for a short while, centrifugalized. For the larger pieces of tissue the small mincing machine devised by Haaland[6] would be equally efficacious.

Certain tissues, such as muscle, were too tough to be cut up by this instrument; they were therefore minced as small as possible with sterile scissors in Ringer's fluid and then centrifugalized.

The experiments were carried out in two groups. In the first group the animal was anesthetized, blood removed from the carotid artery, and the tissue required for making the extract then cut out. Small pieces of the viscera which were to be cultivated were then removed and placed in a sterile bowl of Ringer's fluid. By this means cultures were made of several tissues in the same extract which was autogenous to the tissues. 805 cultures were made in this group.

In the second group an animal was killed on a certain day, several of its organs were removed, and extracts made from them. The extracts were kept on ice for twenty-four hours and then centrifugalized. Subsequently one viscus was removed from another animal and cultured with all the extracts. By this means cultures were made of one tissue in several extracts which were homogenous to the tissue. Two hundred and sixteen cultures were made in this group.

This second group served not only as a control to the first group and thereby decreased the possibility of experimental error, but also served to show whether there was any alteration when homogenous instead of autogenous extracts were used.

[6] Haaland, M., *Berl. klin. Wchnschr.*, 1907, xliv, 713.

The results obtained by the former method will be first considered.

SPLEEN EXTRACT.

In this investigation 238 cultures were made, the effect of the extract being tried on the growth of spleen, thyroid, testicle, liver, and kidney. It was found that the tissues reacted somewhat differently, so that the effects on each tissue must be described.

Spleen.—Of this tissue fifty-eight cultures were made and the results were found to be constant. Even in the early stages the emigration of cells was somewhat more marked in the case of the cultures with the extract than in the controls. At the end of twenty-four hours the number of round cells in the plasma was distinctly greater and they had passed further into the medium. It is doubtful, however, if these cells should be described as parenchymatous cells. They appear to be polynuclear and mononuclear blood cells, and never in any of the preparations have mitotic figures been demonstrable in them, either in the controls or in the preparations with the extracts. The presence of these cells in large numbers to a certain extent obscures the changes of true growth and gives rise to some difficulty in the comparison of any two specimens. They must not be confounded with other cells which appear after two or three days. These are much larger, cuboidal in shape, and grow in solid masses from the edge of the tissues. They have a well defined nucleus with a wide zone of protoplasm. They are probably parenchymatous in nature and arise from the spleen pulp. They are quite distinct from the radiating, branching, and more deeply staining connective cells which on the third or fourth day begin to grow into them from the edge of the tissue. In the preparations to which spleen extract had been added the growth of these cells was always more marked than in the controls, so that whereas on the fourth day specimens grown in simple plasma would often show only irregular patches of these cells the corresponding specimens with the extract would not infrequently show a wide mosaic-like sheet as wide as, or wider than, the original piece of tissue. After four or five days the growth of cells of the connective tissue type was also more advanced in the stimulated specimens. The increase was not very marked, but was constant in all cases. Not only was the

growth of this type of cell more extensive, but it commenced at an earlier date so that they were seen on the second or third day, whilst in the controls they were not definite until the third or even the fourth day. In the subcultures both the parenchymatous and connective cells were more marked in the specimens with the extract for the first subculture, but in the later subcultures the parenchymatous cells were overgrown by the connective tissue type, the growth of which, however, was always more extensive in the specimens containing the extract.

Thyroid.—Fifty-four cultures were made of this tissue. In some of the earlier experiments no growth occurred with the extract, but in all these there was very slight growth in the controls. In the later experiments the growth was much more marked in the preparations with the extract than in the controls. The cells grew rapidly from the edge of the tissue and were mainly of the parenchymatous type, so that after five or six days large solid masses of cuboidal cells were seen projecting into the surrounding medium. Although the greater number of the cells were of the parenchymatous type there was also an increase of the connective tissue cells, these in all cases being more abundant than in the controls. In the case of the controls the majority of the cells were of the connective tissue type, there being very few parenchymatous cells present.

When subcultures were made the cells even of the stimulated specimens became more and more of the connective tissue type. That is to say, when life is prolonged the connective tissue cells, both in stimulated and unstimulated specimens, tend to overgrow the parenchymatous cells (figures 1, 2, and 3).

Testicle.—Of this tissue thirty-six cultures were made and in every case the extract caused marked stimulation of growth. Even after twenty-four hours the difference between the stimulated specimens and the controls was visible. In the case of the controls only a few branching cells were observed spreading from the edge of the tissue, but in the stimulated specimens these formed a well marked ring around the tissue. Growth rapidly progressed and by the fourth day the new tissue formed a mass four or five times the diameter of the original tissue (figure 7). Close to the cut edges the newly growing tissue

formed a solid mass indistinguishable from the original tissue, so that this appeared to have markedly increased in size. This is well shown in the figures of the stimulated and unstimulated specimens, the former of which is only half the magnification of the latter (figures 4 and 7). Outside the solid masses so formed the cells were widely spreading in all directions, so that a mosaic was formed apparently only one cell thick. Two types of cells were discernible: branching, irregular connective tissue cells, and more cuboidal cells with a dark staining nucleus and more distinct protoplasm. These latter cells were always grouped together to form tongue-shaped masses radiating from the sides of the tissue. In the controls the growth of the cells which were apparently parenchymatous in nature was much less marked, nearly all the cells being branching, elongated, and apparently of connective tissue, although even these cells were not growing to nearly so marked an extent as the similar type of cell in the stimulated specimens.

It is interesting to note that with this tissue there was no vacuolation of the plasma. If the cultures were left for a relatively long period without subculturing the cells became granular and degenerate, so that on staining only a mass of debris was seen, but in no case were large vacuoles formed such as are common with degenerate specimens of other tissues.

In the subcultures the connective type of cell rapidly overgrew the parenchymatous in the usual way, but the growth of the connective tissue type of cell was more marked and life could be prolonged to a greater extent in those cases where an extract had been used.

Liver.—Of this tissue thirty-six cultures were also made. In them the variation between the growth of parenchymatous and connective tissue cells is much more evident, for the parenchymatous cells of the liver form solid, deeply staining masses which can readily be distinguished from the connective tissue type. In all cases it was found that in the controls the characteristic feature was the presence of large club-shaped masses of cuboidal cells radiating from the cut edge into the plasma, while the connective tissue cells were present only in small numbers (figure 10). In the specimens to which the extract had been added there was a considerable in-

crease in the growth of the branching spindle cells, but only a few cuboidal cells were present. It is important to note that the parenchymatous cells were not only relatively but actually less in number in the tissues to which the extract had been added. The same characteristics were seen in subcultures which had been fixed in a relatively early stage. In the specimens with the extract there were well defined connective tissue overgrowth and only a few parenchymatous cells, whereas in the controls the amount of parenchymatous growth was extensive and the connective tissue growth less marked. Later, even in the specimens without extract, the cuboidal cells were overgrown by those of the connective tissue type.

Kidney.—Of this tissue fifty-four cultures were made. It was found necessary to exercise caution in drawing conclusions, for of all tissues this seems to be the one most likely to die, and in a series of cultivations several may die from no apparent cause. It was found, however, that in all cases where growth was present this was more marked in the stimulated specimens and that a large proportion of them showed growth. Not only was there an increase of parenchymatous growth, but the connective tissue cells were present also in large numbers. As I have previously shown, the growth of kidney tissue in normal plasma is characterized by the presence of cuboidal parenchymatous cells, very few connective tissue cells being present.

TABLE I.

Effect of Spleen Extract.

Tissue.	With extract.		Without extract.	
	Parenchymatous.	Connective.	Parenchymatous.	Connective.
Spleen . . .	Fair, slightly increased	Very good, considerably increased	Fair	Fair.
Thyroid . .	Very good	Good	Fair	Moderate.
Testicle . .	Very extensive	Extensive	Fair	Good.
Liver	Slight	Good	Very good	Slight.
Kidney . .	Good	Good	Fair	Very slight.

Spleen extract would therefore seem always to stimulate the growth of connective tissue to a considerable extent. Its effect on the parenchymatous cells appears to differ with the tissues used. Of

the organs investigated, growth of these cells was markedly stimulated in the case of the testicle and thyroid, slightly stimulated in the kidney and spleen tissues, and inhibited in the case of the liver. These results are perhaps more clearly shown by table I.

MUSCLE EXTRACT.

In this investigation 259 cultures were made, the same tissues being used as with the spleen extract. As already mentioned, this extract had, owing to the toughness of the tissue, to be made by mincing the tissue with a pair of scissors. For this reason it was probably not so concentrated as were the other extracts, but here, as in other cases, controls were grown in plasma diluted with Ringer's fluid and it was found that the changes about to be described were not due to the fact that the plasma was diluted.

Spleen.—Seventy-eight cultures of this tissue were made. The effect of the extract was noticeable after growth had proceeded for a few hours. Thus after four hours the number of round cells which had emigrated into the plasma was in all cases more marked when the extract had been used. After twenty-four hours this difference was more evident, so that in the cultures with the extract the cells had wandered nearly to the limit of the plasma and formed a large mass around the tissue. The increased emigration of cells is liable to give rise to the belief that growth has been stimulated by the extract, but when care is taken to observe only the cuboidal parenchymatous cells this is seen not to be the case. In the preparations which have had the muscle extract added to them the growth of cuboidal cells is much less, and only a few of these cells are seen at the time when the same cells form a big area in the preparations without extract. On the other hand, the branching connective cells grow more rapidly in those cases in which the extract is added. In the case of the subcultures the preparations without extract show in the first subculture an overgrowth of the cuboidal parenchymatous cells, but those with the extract showed only radiating connective cells. In the third or fourth subculture the connective tissue cells are alone seen in both preparations. It would therefore appear that the muscle extract stimulates the growth of connective tissue cells but inhibits the parenchymatous cells of the spleen.

Thyroid.—Seventy-three cultures of this tissue were made. The results given were very definite. The specimens without the extract showed after two or three days the outgrowth of cuboidal cells which formed a mosaic around the tissue. The extent of the growth of these cells varied considerably with the animals used, but in all cases it was visible. Shortly after their appearance a few branching connective tissue cells were seen which soon grew out as radiating branches. In the case of the preparations with the extract the growth of the cuboidal cells was always much less, and in the majority of cases no such cells could be seen. The growth of the connective tissue cells was, however, much more rapid, so that by the fifth or sixth day a very wide network of branching cells was seen around the original tissue. The distinction between this wide branching network of connective tissue cells and the mosaic of cuboidal cells seen in the preparations without extract and in those treated with spleen extract was very clear.

Testicle.—Thirty-six cultures were made. The effect of this extract was not nearly so marked as that of the spleen. In all cases, however, there was a stimulation of the connective tissue type of cell. The branching cells wandered to a considerable distance into the plasma, forming an open network. The cuboidal parenchymatous cells also showed a distinct overgrowth in the stimulated specimens. In the unstimulated ones only small tongue-shaped processes of these cells were seen, whereas in the ones with the extract it was not uncommon to find a mosaic of these cells as large as the original piece of tissue. This overgrowth was observable both in the primary cultures and in the first subcultures. In the later subcultures these cells were overgrown in the usual way by the connective tissue cells, the growth of which was, as in the primary cultures, more marked in the stimulated specimens.

Liver.—Twenty-four cultures were made. In all cases the preparations containing the muscle extract showed more marked growth of the connective tissue type of cell, but again not to so great an extent as when the spleen extract was used. The effect upon the parenchymatous cells was not so definite. Those preparations with no extract again showed in all cases the characteristic masses of deeply staining cuboidal cells. In the preparations with the extract

these masses were also present, but they were never so extensive, the connective type of cell always preponderating. Similar changes were seen in subcultures. In the first subcultures both types of cell were seen, but the parenchymatous type of cell was more abundant in the specimens without the extract, the connective type in those with the extract. In later subcultures the connective tissue cell was more abundant in both.

Kidney.—Forty-eight cultures were made. As usual, a certain number of specimens died in both preparations. In cases in which growth took place, it was, however, clearly demonstrated that this was more marked in the specimens to which the extract had been added. Not only was the amount of growth more extensive, but in two parallel series those with the extract always showed a larger percentage of successful cultivations. As was to be expected from the results obtained with other tissues, the growth of connective tissue was more marked with the extract, but here again it was not nearly so extensive as when spleen extract had been used. In addition there was a very evident increased activity of the parenchymatous cells. As is usual with this tissue, these cells formed a definite mosaic apparently only one cell thick around the original piece of tissue, but, whereas in the controls these masses were small and projected only from certain points of the tissue, in the stimulated specimens fixed at a corresponding period of time they were much larger and often surrounded the tissue on all sides.

It would appear then that muscle extract stimulates the growth of connective cells whatever type of tissue be used, but this stimulation is not so marked as with spleen extract, although this difference may be due to the fact that the extract is weaker. On the other hand, some parenchymatous cells are stimulated and some are inhibited. It is important to note that the effects upon these parenchymatous cells differ from those of the spleen extract. Thus this type of cell is strongly stimulated in the case of the kidney, only slightly stimulated in the testicle, slightly inhibited in the liver, and more strongly inhibited in the thyroid and spleen. These results are shown in table II.

TABLE II.

Effect of Muscle Extract.

Tissue.	With extract.		Without extract.	
	Parenchymatous.	Connective.	Parenchymatous.	Connective.
Spleen . . .	Slight or absent	Good, increased	Fair	Fair.
Thyroid. .	Very slight	Good	Fair	Moderate.
Testicle. .	Good, slightly increased	Good	Fair	Fair.
Liver. . . .	Good	Good	Very good	Slight.
Kidney . .	Very good	Good	Fair	Very slight.

TESTICLE EXTRACT.

In investigating this extract 160 cultures were made, the same tissues being used as with the previous extracts. As the testicle of the adult rabbit is soft, an extract is readily prepared.

Spleen.—Thirty-six cultures were made. As in the case of the splenic and muscular extracts, the early emigration of cells was more marked than in the control specimens. The cuboidal cells of the parenchyma showed, however, no increase in any of the specimens. These cells were present, but, if anything, their growth was slightly less than that of the controls. In no case was the effect marked. On the other hand, the growth of connective tissue was considerably increased in all specimens. The extract was distinctly more active in this respect than the muscle extract, but was not so powerful as that of the spleen.

Thyroid.—Twenty cultures were made. In every case there was an increase in the growth of the parenchymatous cells, but not to so marked an extent as when the spleen extract was used. In all there was overgrowth of the connective tissue as compared with the controls. In the subcultures the connective tissue was also more marked when the extract was used, and more rapidly overgrew the parenchymatous cells.

Testicle.—Forty-eight cultures were made. As in the case of the other tissues, there was a considerable increase in the growth of the connective tissue, but never to so marked an extent as when the spleen extract was used. The growth of parenchymatous cells is also distinctly increased, these cells forming large mosaic-like areas when the corresponding controls showed small areas projecting

from the edge of the tissue (figure 8). Not uncommonly by the third day these masses of cuboidal cells formed in the stimulated specimens an area with a diameter as large as, or larger than, that of the original tissue. In no case, however, were these masses nearly so extensive as in the case of the preparations stimulated with splenic extract. In the subcultures the cuboidal cells were in the usual way rapidly overgrown by the connective tissue cells.

Liver.—Thirty-six cultures were made. In all cases there was some increase of the connective tissue cells, these cells being present at an earlier date and growing more rapidly. The parenchymatous cells were definitely decreased in numbers, there being only slight growth of small cuboidal masses from the edge of the tissue (figure 14). The same increased growth of the connective tissue was seen in the subcultures.

Kidney.—Twenty cultures were made. In all cases the tendency for vacuolation which is so marked with this tissue was more in evidence when the extract was used, and in such cases very little growth was seen. If, however, this vacuolation occurred early the cells might continue to grow in the plasma, and owing to the vacuole, were then completely separated from the original tissue. Under such circumstances no connective tissue cells were to be seen, it being probable that the cells are separated from the tissue before this type of cell has commenced to grow. If the growing portion remained attached to the original tissue there was always an increase in the numbers of connective tissue cells in those specimens with the extract. In all cases the growth of parenchymatous cells was much less in the specimens with the extract than in those without it.

Testicular extract appears, then, to stimulate the growth of connective tissue in all cases. This stimulation is more marked than is the case with muscle extract, but is not so extensive as when spleen extract is used. Certain of the parenchymatous cells are stimulated and some of them are inhibited. Thus the growth of this type of cell was always more marked in the case of the testicle and thyroid, was diminished in the case of the kidney and liver, and was but slightly if at all affected in the case of the spleen. These results are shown in table III.

TABLE III.

Effect of Testicle Extract.

Tissue.	With extract.		Without extract.	
	Parenchymatous.	Connective.	Parenchymatous.	Connective.
Spleen..........	Fair	Good	Fair	Fair.
Thyroid........	Good	Good	Fair	Moderate.
Testicle.......	Very good	Good	Fair	Fair.
Liver......	Very slight	Good	Very good	Slight.
Kidney.........	Slight	Good	Fair	Very slight.

THYROID EXTRACT.

In the investigation of this extract 148 cultures were made, the same tissues being again used. Owing to the small size of the thyroid gland, only small quantities of the extract could be prepared from each animal.

Spleen.—Sixteen cultures of this tissue were made. As with the other extracts, the specimens to which this extract was added showed an increase in the number of cells emigrating in the early stages. The growth of both parenchymatous and connective tissue cells was also definitely increased. By the third day the specimens with the extract already showed many branching connective tissue cells and masses of cuboidal parenchymatous cells, the corresponding cells being much less marked in the controls. By the fifth day the specimens with the extract were surrounded by large masses of parenchymatous cells, while close to the edge of the original tissue the connective cells formed a branching network difficult to distinguish from the tissue itself.

Thyroid.—Thirty-four cultures were made. The connective tissue cells were again increased, but not so extensively as when spleen extract was used, the amount of growth of this type of cell resembling that found when testicular extract was used. The parenchymatous cells were also somewhat more in evidence than in the controls, but the amount of stimulation of these cells was slight. The growth was not nearly so extensive as when splenic extract was used, and in fact in some cases it was but little more marked than in the controls.

Testicle.—Forty cultures were made. In all the experiments

there was a very marked increase in the growth of the parenchymatous cells. The growth of these cells was as extensive as with the use of splenic extract, so that by the fourth or fifth day large masses of these cuboidal cells were seen extending widely from the edge of the tissue into the surrounding medium. The connective tissue cells were, however, as in the case of the other tissues, not stimulated to nearly so marked an extent as by the spleen extract, although the growth was always more extensive than in the case of the controls. For this reason the masses of cuboidal cells were more clearly visible than when spleen extract was used (figure 6). In the subcultures the connective tissue grew more rapidly, so that the growth of the parenchymatous cells was not nearly so marked.

Liver.—Thirty-six cultures were made. The connective tissue growth was again increased to a moderate extent. The characteristic masses of deeply staining cuboidal cells were also more extensive with the use of the extract, the specimens in this particular differing markedly from those in which splenic extract was used (figure 12). The cuboidal cells formed large rounded or club-shaped masses growing irregularly from the edge of the tissue, but in some cases where the medium was thinner they formed mosaic-like masses apparently only one cell thick, which completely surrounded the original tissue and extended for some distance into the medium around. In the subcultures the connective tissue growth was more marked.

Kidney.—Twenty-two cultures were made. In these specimens again there was some increase in the connective tissue growth. The amount of growth of the parenchymatous cells was always less extensive than in the case of the controls. There was also a greater tendency for the liquefaction of the medium to take place, the original tissue being thereby separated by a fluid ring from the growing cells.

Thyroid extract appears, therefore, always to stimulate the growth of the connective tissue type of cell, whatever tissue is used, the amount of stimulation being about equal to that with testicular extract, but being less marked than that found with the use of splenic extract. Of the different tissues employed in this inves-

tigation the majority showed an increase in the growth of the parenchymatous cells when the extract was added. The extent of this stimulation differed, however, with the various cells. Thus, whereas this type of cell was markedly stimulated in the case of the spleen, testicle, and liver, it was only slightly increased in the case of the thyroid, and inhibited in the case of the kidney. These results are shown in table IV.

TABLE IV.

Effect of Thyroid Extract.

Tissue.	With extract.		Without extract.	
	Parenchymatous.	Connective.	Parenchymatous.	Connective.
Spleen..........	Very good	Good	Fair	Fair.
Thyroid........	Good	Good	Fair	Moderate.
Testicle.........	Very good	Good	Fair	Fair.
Liver...........	Very good	Good	Very good	Slight.
Kidney.........	Slight	Good	Fair	Very slight.

In the second group of experiments one tissue only was cultivated from each animal. The extracts had been previously prepared from the tissues of another animal, as already described. The fluid was pipetted off the emulsified tissue and kept on ice in a sterile tube. In some cases it was used after twenty-four hours, but in other cases only after several days had elapsed. In this way it was possible to determine whether the effects of the extract were in any way altered by preserving it on ice. It will be seen that in all the experiments in this group the extracts were homogenous to the tissues, whereas in the last group they were autogenous. In no case was it evident that the homogenous extracts acted in any way differently from the autogenous extracts. The homogenous extracts were as efficient as the autogenous.

In this group 216 cultures were made.

Thyroid.—The thyroid was removed from a rabbit, and thirty-six preparations were made and cultivated in groups of six each in the following media: plasma, plasma and Ringer's fluid 2 to 1, plasma and thyroid extract 2 to 1, plasma and spleen extract 2 to 1, plasma and testicular extract 2 to 1, plasma and liver extract 2 to 1. All the extracts were one day old.

The results of these cultivations confirmed those made in the previous group. The controls showed a fair amount of growth of both the connective and parenchymatous cells, but with Ringer's fluid there was a slight increase in the growth of the connective tissue. The preparations with the thyroid and testicular extracts both showed a slight increase of the parenchymatous and connective tissue cells, while those with the spleen extract showed a considerable increase of both varieties of cells. Those with the liver extract showed, however, a marked diminution both in the parenchymatous and the connective tissue cells.

Testicle.—The testicle was removed and thirty-six preparations were made. The same culture media were used as in the last experiment, the extracts in this case being eleven days old. Here again the results confirmed those obtained in the first group of experiments. The control specimens showed a fair amount of parenchymatous and connective tissue growth (figure 4). With Ringer's fluid there was a definite increase in the amount of connective tissue growth although the parenchymatous cells were not increased (figure 5). With the thyroid extract there was a marked increase in the amount of parenchymatous growth and a slight increase in the connective tissue growth (figure 6), a similar result being obtained with the testicular extract (figure 8). With the splenic extract both the parenchymatous and connective tissue cells were greatly increased (figure 7). With the liver extract, as in the case with the thyroid tissue, there was no growth of the parenchymatous, and a greatly diminished growth of the connective tissue cells (figure 9).

A second experiment, performed on precisely the same lines, gave identical results, the extracts in this case being thirteen days old.

Kidney.—The kidney was removed and thirty-six preparations were made in groups of six specimens in each of the same culture media as before. The results agreed with those of the first group. In the controls there was good growth mainly of the parenchymatous cells, very few of the connective cells being present. In the specimens with Ringer's fluid the growth of connective cells was

rather more marked, but with this diluent the medium liquefied more readily and hence the growth of parenchymatous cells was less. With the thyroid extract the growth of parenchymatous cells was decreased as compared with the controls, but that of the connective cells was somewhat more marked. A similar result was obtained with the use of testicular extract. With the spleen extract there was a very definite increase in the amount of growth of both types of cell, but with the liver extract again there was very little or no growth.

Liver.—Thirty-six preparations were made in the same varieties of media, the extracts in this case being one day old. Good growth of both parenchymatous and connective tissue cells was obtained in the controls (figure 10). With Ringer's fluid the growth of the connective tissue cells was increased, but that of the parenchymatous cells was somewhat lessened (figure 11). With thyroid extract the connective growth was increased to a moderate extent, while the parenchymatous growth was considerably increased (figure 12). Testicular extract had a similar effect upon the connective cells, but diminished the growth of the parenchymatous cells (figure 14). This action is of interest, for in all the tissues previously considered these two extracts had a similar effect, but in the case of the liver tissue the one inhibits and the other stimulates the growth of the parenchymatous cells. With liver extract the growth of both types of cell was considerably diminished (figure 15). Splenic extract stimulates only the connective tissue growth (figure 13).

A second experiment was carried out on similar lines, but in this case the extracts were all twenty days old. It was found that in this case no growth occurred with any of the extracts, although good growth was seen in the preparations in simple plasma and in those in plasma and Ringer's fluid.

The results, therefore, of this series of experiments confirmed those of the first group, but in addition it was seen that liver extract apparently strongly inhibits the growth of both the connective tissue and parenchymatous cells of all tissues. The effects of other extracts are constant whether they are homogenous or autogenous

to the tissue used. Up to a certain period of time the extracts can be kept unchanged on ice, but after this time they appear to undergo a change.

CONCLUSIONS.

The cultivation of cells *in vitro* affords a valuable means of estimating the effects of tissue extracts.

Tissue extracts have a definite effect upon the growth of adult mammalian cells *in vitro.*

The majority of tissue extracts stimulate the growth of connective tissue, but liver extract inhibits it.

The extracts are to a certain extent specific in their action upon the growth of parenchymatous cells. Some cells are stimulated by one extract and inhibited by another, and those extracts which inhibit one type of parenchymatous cell may stimulate another type.

Homogenous and autogenous extracts are equally efficacious in their action upon the growth of cells.

The extracts may be preserved for a short period of time without suffering any change in their power of affecting the growth of cells.

EXPLANATION OF PLATES.[7]

Plate 27.

Growth of adult rabbit thyroid in plasma and spleen extract.

Fig. 1. First culture. Fourth day.
Fig. 2. Subculture. Third day.
Fig. 3. Second subculture. Third day.

Plate 28.

Growth of adult rabbit testicle in various media.

Fig. 4. Four days' growth in simple plasma.
Fig. 5. Four days' growth in plasma and Ringer's fluid.
Fig. 6. Four days' growth in plasma and thyroid extract.

Plate 29.

Growth of adult rabbit testicle in various media.

Fig. 7. Four days' growth in plasma and splenic extract.
Fig. 8. Four days' growth in plasma and testicular extract.
Fig. 9. Four days' growth in plasma and liver extract.

[7] I am indebted to Mr. Summers for the photographs of the growing tissues.

PLATE 30.

Growth of adult rabbit liver in various media.

FIG. 10. Four days' growth in simple plasma.
FIG. 11. Four days' growth in plasma and Ringer's fluid.
FIG. 12. Four days' growth in plasma and thyroid extract.

PLATE 31.

Growth of adult rabbit liver in various media.

FIG. 13. Four days' growth in plasma and splenic extract.
FIG. 14. Four days' growth in plasma and testicular extract.
FIG. 15. Four days' growth in plasma and liver extract.

OBSERVATIONS ON EXPERIMENTAL TYPHOID INFECTION OF THE GALL BLADDER IN THE RABBIT.*

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In recent years the question of immunity following antityphoid vaccination, the typhoid carrier problem, and the possibilities of chemotherapy have restimulated investigation of experimental typhoid infections in the lower animals.

A review of the present status of infection in animals below the apes is given by Hailer and Ungermann (1). In the case of the rabbit the original observation dates back to 1891, when Blachstein and Welch (2) noted that, after intravenous inoculation of colon and typhoid bacilli, rabbits may develop a lesion of the gall bladder which may persist for several months. Apparently no further work was done with this lesion at that time, but, in the last few years, infection of the organs of the rabbit and especially of the gall bladder have been used for various purposes by Doerr (3), Koch (4), Chiarolanza (5), Conradi (6), Hailer and Rimpau (7), Hailer and Ungermann (8), Hailer and Wolf (9), Uhlenhuth and Messerschmidt (10), Morgan (11), Johnston (12), Gay and Claypole (13), and Cummins and Cumming (14).

It has been shown that typhoid bacilli can be isolated from the organs for some time after injection, but that the gall bladder lesion is the most persistent source. After intravenous injection, the gall bladder apparently becomes infected by way of the blood stream as well as from the bile. It has also been shown that intravenous inoculation does not regularly result in producing gall bladder lesions and most of the experimenters in chemotherapy have given up this method for the direct inoculation of the gall bladder. The intravenous method of infection, however, more nearly resembles the natural method and has advantages for some purposes.

In this paper the following subjects are considered: (1) patho-

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genicity of a living sensitized vaccine, (2) pathogenicity of first transplant from a living sensitized vaccine, (3) the regular production of lesions, (4) the gall bladder lesion as a test of immunity, (5) the curative effects of vaccines, and (6) the practical bearing of experimental work. Altogether, ninety-seven animals have been used and forty gall bladder lesions have been observed.

PATHOGENICITY OF A LIVING SENSITIZED VACCINE.

The writer's attention was first attracted to the gall bladder lesion as a means of determining whether or not a living sensitized vaccine is pathogenic. As is well known, Metchnikoff and Besredka (15), as a result of their work with experimental typhoid in the chimpanzee, devised a living sensitized vaccine for protection. This vaccine has been criticized as likely to produce carriers, if not the disease itself. Metchnikoff and Besredka's own observations on the possibility of producing carriers in apes and man have been negative. My own experience with rabbits has been the same. Through the kindness of Prof. Besredka I secured some of his vaccine which was living at the time of use, as evidenced by the growth from the vaccine of a typical typhoid organism. The vaccine was given intravenously to nine animals, and subcutaneously to one animal, in doses of two to six cubic centimeters. Two animals were sacrificed on the day following intravenous injection, and cultures made from the bile were sterile. The other animals were examined after one month; cultures from the bile were negative in all cases for typhoid bacilli, and no macroscopic evidence of disease was found. It may, therefore, be concluded that intravenous injection of this vaccine does not produce gall bladder lesions in the rabbit, and by inference that no danger exists of producing carriers in man by subcutaneous injection.

PATHOGENICITY OF THE FIRST TRANSFER OF A LIVING SENSITIZED VACCINE.

While the original vaccine is apparently harmless, the first transplant on agar is pathogenic, as is indicated by the following experiment.

Nov. 18, 1913. A large agar slant was inoculated with vaccine and, after twenty-four hours, the growth was collected in 20 c.c. of salt solution and 6 c.c. were given intravenously to a rabbit weighing 3,000 gm. On Dec. 23, the animal was killed and a well marked lesion of the gall bladder was found and the bile contained a pure culture of typhoid bacilli.

Hence while the vaccine itself is apparently safe, it has possibilities of harm in case it became accidentally deposited on media suitable for multiplication. This is a point of some practical importance in the military service in which ampules may unavoidably be broken in transportation or in camp. According to the results of this experiment, any water or food accidentally contaminated with this vaccine might transmit the disease to non-immunes. It may, therefore, be stated that a living sensitized vaccine is not entirely safe to handle, as is a killed vaccine (16).

ATTEMPTS TO PRODUCE GALL BLADDER LESIONS REGULARLY BY INTRAVENOUS INJECTION.

While the above work was in progress, Gay and Claypole (13) published a paper in which the authors stated that regular infections had been produced by intravenous injection of growths from rabbit blood agar. In order to try the effect of this procedure, the strain from the living vaccine was carried on rabbit blood agar and passed through lesions in five animals, as is seen in table I. The growth on a blood agar slant was suspended in twenty cubic centimeters of salt solution, and two cubic centimeters, or one half the fatal dose, were given intravenously and the animals were examined after one month.

TABLE I.

Attempts to Produce Gall Bladder Lesions with Transfers from Besredka's Vaccine on Blood Agar.

No. of passage.	No. of animals.	Positive.	Negative.
1	1	1	0
2	6	1	5
3	3	1	2
4	1	1	0
5	5	1	4
	16	5 (31%)	11

It will be seen that with this strain cultivation on blood agar had no effect in maintaining pathogenicity, nor did passage through four animals in series. In the fifth transfer only one animal out of five became infected. In this connection it should be noted that the strain Gay and Claypole worked with was recently isolated.

In order to determine the effect of the age of the culture several strains were tried with the following results. One half the fatal dose of an agar or bouillon growth was given intravenously and the animals were examined after one month (table II).

TABLE II.

Attempts to Produce Gall Bladder Lesions with Various Strains Given Intravenously.

Strain.	Age of strain.	No. of animals.	Positive.	Negative.
Clark	1 mo.	8	6	2
Fuguet	1 mo.	2	1	1
Andrews	1 mo.	4	0	4
Clark	3 mos.	4	0	4
Rawlings	14 yrs.	6	0	6
Dorset	15 yrs.	5	1	4
Besredka	?	16	5	11
		45	13 (28.8%)	32

The conclusion to be drawn from these experiments is that freshly isolated strains produce lesions in a higher percentage than old strains, but even freshly isolated strains cannot always be depended on to produce lesions by the intravenous method. This conclusion agrees with that of nearly every investigator and has been emphasized by Uhlenhuth and Messerschmidt, and Hailer and Ungermann. As stated above, these authors have given up the intravenous method for the more certain method of direct inoculation of the gall bladder.

THE GALL BLADDER LESION AS A TEST OF IMMUNITY.

Gay and Claypole also state that the gall bladder lesion can be used as a test of immunity conferred by vaccines. Evidently such a test in an animal would be of great value in many ways, but unfortunately this lesion does not seem to be suitable for such a

purpose. The instantaneous introduction of a large number of bacilli directly into the circulation seems to break down any artificial immunity produced by vaccines. Uhlenhuth and Messerschmidt (10) have been unable to protect animals from infection with the Pfeiffer-Kolle vaccine, although immune bodies were present in large quantities. The infecting dose was given directly into the gall bladder. My experience has been the same with the Army vaccine, using the intravenous route. Although it is impossible to carry on well controlled experiments with the intravenous method when the results are so uncertain, the positive results have some significance.

Ten rabbits were used. Six were vaccinated with 100 million subcutaneously, followed at weekly intervals by two doses of 200 million, intravenously. Four were vaccinated similarly with doses of 500 and 1,000 million. Ten days after the last vaccination, all were given an intravenous injection of about one half the fatal dose of Besredka's organism. After one month, two animals of each set showed definite infections of the gall bladder. The percentage of infections was 40, while the percentage of infections among controls (16) was 31.

Hence no protective effect of vaccination could be demonstrated in the rabbit. As the protective effect of the same vaccine in man is well known, this result simply means that the conditions of the experiment in the rabbit are not comparable with those in natural infections in man.

ATTEMPTS TO CURE THE GALL BLADDER LESION BY VACCINES.

Johnston (12) states that vaccines may have some curative effect on the lesion of the gall bladder. Uhlenhuth and Messerschmidt (10) were unable to obtain such a result, and Cummins and Cumming (14) also state that vaccine treatment did not influence the finding of living bacilli in the liver and gall bladder. I have also been unable to demonstrate any curative effect of vaccines.

Two rabbits were given 1 c.c. of a broth culture of Clark strain intravenously on Apr. 27, 1914. Plating of feces was positive to typhoid bacilli on May 15, 1914. Both animals were then given subcutaneously four doses of vaccine, one dose every five days, running from 10 to 80 million. One week after the last dose the animals were autopsied, and the bile in both cases yielded a pure culture of typhoid bacilli.

Two human carriers who have been studied in this laboratory and reported by Leary (17) were treated over several months with autogenous and stock vaccines and failed to show any marked diminution in the excretion of bacteria. Both cases were operated on and the gall bladders, containing pure cultures of the organism, were removed. Excretion of typhoid bacilli in the stools ceased permanently in nine days. It is, of course, not claimed that removal of the gall bladder will cure all carriers, because we know that the bile passages and intestinal mucosa may be affected in some cases. But our experience with the curative effect of vaccines has not been encouraging either clinically or experimentally.

PRACTICAL BEARING OF EXPERIMENTAL WORK.

In this laboratory our chief interest in experimental infections has naturally centered in their possible relation to immunity following vaccination. As is well known, the present army vaccine was devised in 1908 by Major F. F. Russell of the Medical Corps, on the basis of German and English experience. During the last five years we have manufactured over 1,700,000 cubic centimeters for the Army, Navy, National Guard, and other departments of the Government, and over 250,000 men have been immunized. The results of the use of this product have been fully detailed by Major Russell (18) in a number of contributions to our knowledge of this subject. It is only necessary here to emphasize the fact that the conditions of its use have been those of extensive experimentation. The subjects are exposed to infection in many parts of the world and are under close observation for several years at least. The results have exceeded all expectations, and, on the basis of English and American experience, we can now give a definite answer to some of the problems of immunity in man.

In the first place it is clear that the conclusions reached by Metchnikoff and Besredka from their work on chimpanzees cannot be accepted for man. These authors found that a whole killed vaccine did not protect their animals. But they used tremendous infecting doses,—the contents of a whole Kolle flask. On the other hand, they were able to protect with a living sensitized vaccine. But, as

has already been pointed out, this vaccine is not entirely safe to handle and its use could certainly not be made compulsory. Hence, if we followed these experimental results strictly, we would not be using any vaccine and the splendid results would have been lost. Inasmuch as the final object of all experimental work on this subject is the protection of man, it would be a *reductio ad absurdum* to give up the goal, once reached, because we cannot duplicate the conditions in animals.

In the same way the gall bladder lesion in the rabbit fails as an index of immunity from vaccination in relation to man. For an attack on the typhoid carrier problem this lesion seems ideal, and it may possibly serve in advancing the chemotherapy of typhoid fever. It certainly gives an admirable experimental opportunity for testing methods of isolating typhoid bacilli from the feces. But the claim of Gay and Claypole, that this lesion can be used as a test of immunity from vaccination, seems unfounded, and for the same reason as in the case of the chimpanzees. Human conditions cannot be duplicated and immunization which is known to protect man is not effective under these different conditions.

While the subject was developing, many products were proposed; the whole killed vaccine, autolysates, residues, fractions, etc. When it became evident that a whole killed vaccine had some advantages, other questions arose. What strain should be used? Should it be virulent or avirulent? Should it be fresh or old? Should several strains be used? Should they be sensitized? A great deal of work done with immune bodies has given no convincing answer to these questions. As a result of actual trial we can say that a whole vaccine killed by heat at 53° C. and preserved with 0.25 per cent. tricresol protects human beings from typhoid fever; that an old avirulent strain is effective; that a single strain is effective against typhoid in all parts of the world (19); that doses of 500 and 1,000 million at intervals of seven or ten days are effective; that two doses protect for two years and that three doses protect for a longer period not yet exactly determined; that the vaccine keeps its immunizing properties for at least four months.

Gay and Claypole say that "Empiricism rather than experimentation has largely determined the method employed." On the con-

trary, experimentation has marked every step of the evolution of our present vaccine since the first paper on the subject in 1896, by Pfeiffer and Kolle (20). The only peculiarity has been that the teachings of clinical experience have been given some weight; and that experimental results have been taken for just what they were worth and no more. No claim is made that the Army vaccine is the *summum bonum* in antityphoid vaccines. Improvements may come, but before our results are improved upon, they must be equalled. And they must be equalled, not in a few chimpanzees or rabbits, but in large numbers of human beings under observation for several years.

At present the subjects which give concern are, naturally, the length of immunity and the keeping qualities of the vaccine. Evidence on these points is accumulating from actual experience, and we know of no other safe method of reaching conclusions on these points.

SUMMARY.

1. Besredka's living sensitized vaccine, given intravenously, does not produce a typhoid lesion of the gall bladder in the rabbit.

2. The first transplant of this vaccine is capable of producing this lesion. Hence this vaccine is not entirely safe to handle.

3. Regular infections of the gall bladder have not been produced by carrying a known pathogenic strain on rabbit blood agar, by successive passage through animals, or by the use of freshly isolated strains.

4. No evidence could be demonstrated in the rabbit of the immunity produced in man by vaccination with a whole killed vaccine.

5. Vaccine treatment did not cure the gall bladder lesion.

6. With the present methods of producing infections in the chimpanzee and the rabbit, neither of these animals is suitable for deciding the problems of the immunization of man by vaccines. These problems must be settled, as some of them already have been settled, by actual experience with large numbers of men kept under close observation.

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PROTEOTOXINS (ANAPHYLATOXINS) AND VIRULENCE.*

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I.

In a series of experiments reported in another paper (1), the writers have shown that guinea pigs, previously injected with sub-lethal doses of bacterial proteotoxin (anaphylatoxin), may acquire slightly but definitely increased tolerance to these poisons. In the course of this work the material used for injection consisted of the supernatant fluid remaining after vigorous and prolonged centrifugation of mixtures of typhoid bacilli and fresh guinea pig serum. Although centrifugation was sometimes continued for two hours and more, the process never entirely freed the serum of bacteria, in spite of the fact that a settling of the microörganisms was often facilitated by the presence of normal agglutinins in the guinea pig serum, and, occasionally, by the addition of small amounts of inactivated, strongly agglutinating antityphoid serum.

The filtration of the preparation through Berkefeld candles freed it of bacteria, but, as reported in a previous paper, also entirely removed the toxic properties, and for this reason such a procedure could not be utilized.

The fluids obtained were always perfectly clear and showed no cloud on shaking, but on cultural examination, in which several loopfuls were planted, a few colonies always appeared. The majority of the animals injected with these fluids, if they survived acute shock, went on to complete recovery. Not infrequently, however, a guinea pig that had recovered entirely from the immediate effects of the poison would die, in the course of two or three days, of typhoid infection, cultures from the heart's blood showing typhoid bacilli abundantly present.

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Since the minimal fatal dose of our typhoid culture for guinea pigs of about 200 grams was one tenth to one fifteenth of an agar slant, and it was plain that nothing like this amount could have been injected with the two or three cubic centimeters of proteotoxin (anaphylatoxin) in the animals described above, it seemed as though the poison itself might have possessed aggressin-like properties by virtue of which the sublethal amounts of bacilli, remaining in the serum after centrifugation, had been rendered more powerfully pathogenic.

Our first experiments were aimed purely at the determination of this point. The procedure in these preliminary experiments consisted in determining the minimal lethal dose of the bacteria in question for guinea pigs of a certain weight, and then injecting smaller quantities of bacteria together with sublethal doses of the proteotoxin (anaphylatoxin).

The proteotoxin was prepared throughout with typhoid bacilli, and the bacilli, in proportions of one half agar slant to four cubic centimeters, were digested for five or six hours at 37.5° C. with fresh normal guinea pig serum. Centrifugation was then carried on for one to two hours, cultures were taken, and this fluid was added to the quantities of bacteria measured for the experiment.

EXPERIMENT A.

Typhoid Bacilli Injected Intraperitoneally with Proteotoxin (Anaphylatoxin).

No. of animal.	Weight.	Dose, agar slant.	Proteotoxin (anaphylatoxin).	Salt solution.	Result.
1	200 gm.	1/100	3 c.c.	——	Died in 22 hrs.
2	215 gm.	1/100	3 c.c.	——	Died in 18 hrs.
3	210 gm.	1/200	3 c.c.	——	Died in 18 hrs.
4	215 gm.	1/200	3 c.c.	——	Died in 35 hrs.
5	215 gm.	1/300	3 c.c.	——	Remained alive.
6	215 gm.	1/10	——	3 c.c.	Died in 52 hrs.
7	205 gm.	1/20	——	3 c.c.	Died in 18 hrs.
8	225 gm.	1/50	——	3 c.c.	Remained alive.

It is here seen that 1/200 c.c. of an agar slant was fatal if injected with 3 c.c. of the proteotoxin, whereas with salt solution alone 1/20 was the minimal lethal dose. 3 c.c. of the anaphylatoxin alone did not kill. The same result is shown in the following protocol.

EXPERIMENT B.

Typhoid Bacilli Injected Intraperitoneally with Proteotoxin (Anaphylatoxin).

No. of animal.	Weight.	Dose, agar slant.	Proteotoxin (anaphylatoxin).	Salt solution.	Result.
1	225 gm.	1/100	2.5 c.c.	——	Died in 3 dys.
2	220 gm.	1/200	2.5 c.c.	——	Died in 4 dys.
3	230 gm.	1/10	——	2.5 c.c.	Sick, recovered; remained alive.
4	235 gm.	1/20	——	2.5 c.c.	Remained alive.
5	230 gm.	——	3.0 c.c.	——	Not sick; remained alive.

These experiments show that, when injected intraperitoneally, the proteotoxin enhances the virulence of the typhoid bacilli. It should be noted that, in the case of typhoid infection, these protocols represent unusually favorable results. Although distinct differences pointing in the same direction were noticeable in all the experiments, these two represent the best results obtained. More regular results were obtained when the injections were made intravenously.

EXPERIMENT C.

Typhoid Bacilli Injected Intravenously with Proteotoxin (Anaphylatoxin) Produced with Typhoid Bacilli. 3.5 Cubic Centimeters of the Anaphylatoxin Used Killed a Guinea Pig Weighing 240 Grams in Three Minutes with Acute Shock.

No. of animal.	Dose.	Proteotoxin (anaphylatoxin).	Salt solution.	Result.
1	1/40	1.5 c.c.	——	Very sick, but recovered.
2	1/100	1.5 c.c.	——	Died in 48 hrs.; typhoid bacilli in heart's blood.
3	1/200	1.5 c.c.	——	Sick, but recovered.
4	1/10	——	1.5 c.c.	Sick, but recovered.
5	1/20	——	1.5 c.c.	Remained well.
6	1/40	——	1.5 c.c.	Remained well.
7	——	2.0 c.c.	——	Remained well.

This experiment again illustrates the aggressive effect of the poison, but here as in several other experiments irregularities occurred in that the largest dose given with the poison failed to kill, while a smaller dose was fatal (guinea pigs 1 and 2). We cannot explain this and can attribute it at present only to individual differences in the animals, although it occurred a number of times.

In all the preceding cases the proteotoxin was produced with typhoid bacilli, and typhoid bacilli were used in the experiment. The question naturally arose as to whether this aggressive action of the proteotoxin was specific or whether proteotoxin produced

with typhoid bacilli would show the same action when injected with other bacteria. This was a natural question, especially since all recent work has seemed to indicate that the proteotoxins were functionally alike whatever the method used for their production. For this reason the following experiments were carried out in which proteotoxin produced with typhoid bacilli was combined with staphylococci, and in one case with prodigiosus bacilli.

EXPERIMENT D.

Staphylococci Injected Intraperitoneally with Proteotoxin (Anaphylatoxin) Produced with Typhoid Bacilli.

No. of animal.	Dose, agar slant.	Proteotoxin (anaphylatoxin).	Salt solution.	Result.
1	1/100	2 c.c.	——	Died in less than 18 hrs.
2	1/200	2 c.c.	——	Died in less than 18 hrs.
3	1/400	2 c.c.	——	Died in 20 hrs.
4	1/400	2 c.c.	——	Died in less than 18 hrs.
5	1/10	——	2 c.c.	Remained alive.
6	1/50	——	2 c.c.	Remained alive.
7	1/100	——	[illegible] c.c.	Remained alive.
8	——	3 c.c.	——	Died in 5 dys. with typhoid bacilli in heart's blood.

Staphylococci were found in the heart's blood of guinea pigs 2 and 3, the only ones of the series that were autopsied.

EXPERIMENT E.

Staphylococci Injected Intraperitoneally with Proteotoxin (Anaphylatoxin) Produced with Typhoid Bacilli.

No. of animal.	Weight.	Dose, agar slant.	Proteotoxin (anaphylatoxin).	Salt solution.	Result.
1	250 gm.	1/100	3 c.c.	——	Died in 18 hrs.
2	230 gm.	1/100	3 c.c.	——	Died in 16 hrs.
3	250 gm.	1/200	3 c.c.	——	Died in 22 hrs.
4	225 gm.	1/200	3 c.c.	——	Died in 21 hrs.
5	250 gm.	1/400	3 c.c.	——	Died in 18 hrs.
6	215 gm.	1/800	3 c.c.	——	Died in 29 hrs.
7	175 gm.	1/2	——	3 c.c.	Died in 19 hrs.
8	220 gm.	1/5	——	3 c.c.	Died in 14 hrs.
9	210 gm.	1/10	——	3 c.c.	Remained alive.
10	235 gm.	1/20	——	3 c.c.	Remained alive.
11	240 gm.	——	3 c.c.	——	Died in 3 dys.

Autopsies on guinea pigs 5 and 6 showed pure cultures of staphylococci in the heart's blood. No. 11 showed typhoid bacilli in the heart's blood.

EXPERIMENT F.

Prodigiosus Bacilli Injected Intraperitoneally with Proteotoxin (Anaphylatoxin) Produced with Typhoid Bacilli.

No. of animal.	Dose, agar slant.	Proteotoxin (anaphylatoxin).	Salt solution.	Result.
1	1/50	2 c.c.	——	Died in 18 hrs.
2	1/100	2 c.c.	——	Died in 18 hrs.
3	1/100	2 c.c.	——	Died in 18 hrs.
4	1/200	2 c.c.	——	Died in 18 hrs.
5	1/200	2 c.c.	——	Died in 20 hrs.
6	1/10	——	2 c.c.	Remained alive.
7	1/20	——	2 c.c.	Remained alive.
8	1/40	——	2 c.c.	Remained alive.
9	1/80	——	2 c.c.	Remained alive.
10	——	3 c.c.	——	Died in 5 dys.

Prodigiosus bacilli were found in the heart's blood of guinea pig 1, the only one of this series autopsied for this purpose. In guinea pig 10, the proteotoxin control, death ensued in five days, from generalized typhoid infection due to the few organisms remaining in the fluid after centrifugation. Without invalidating the present series, therefore, this forms an additional corroboration of the point made in experiments A and B.

EXPERIMENT G.

Staphylococci Injected Intravenously with Proteotoxin (Anaphylatoxin) Produced with Typhoid Bacilli.

No. of animal.	Weight.	Dose, agar slant.	Proteotoxin (anaphylatoxin).	Salt solution.	Result.
1	125 gm.	1/40	1 c.c.	——	Died in 2 dys.
2	185 gm.	1/80	1 c.c.	——	Died in 2 dys.
3	160 gm.	1/10	——	1 c.c.	Not sick.
4	190 gm.	——	2 c.c.	——	Slight shock, but recovered and remained alive.

Blood cultures taken from the heart's blood of guinea pigs 1 and 2 just before and after death showed staphylococci in pure culture.

EXPERIMENT H.

Staphylococci Injected Intravenously with Proteotoxin (Anaphylatoxin) Produced with Typhoid Bacilli.

No. of animal.	Weight.	Dose, agar slant.	Proteotoxin (anaphylatoxin).	Salt solution.	Result.
1	185 gm.	1/20	1 c.c.	——	Died in 48 hrs.
2	195 gm.	1/50	1 c.c.	——	Died in 48 hrs.
3	180 gm.	1/10	——	1 c.c.	Remained alive.
4	175 gm.	——	2 c.c.	——	Slight shock, but recovered and remained alive.

Staphylococci and typhoid bacilli, the latter in relatively small number, were recovered in blood plates made of the heart's blood of guinea pigs 1 and 2. The fact that the proteotoxin control, guinea pig 4, was able to overcome completely the typhoid bacilli present in twice the amount of proteotoxin indicates that the staphylococci in this case conversely aided the typhoid infection. The significance of this fact in connection with mixed infections in man will be discussed in our conclusions.

It is noticeable throughout these experiments that, although distinct enhancement of the virulence of typhoid bacilli is accomplished by the addition of the proteotoxin, the results are much sharper and more convincing when staphylococci are the infecting agents. We are inclined to attribute this to the fact that proteotoxin is much more easily formed by the aid of typhoid bacilli than with staphylococci. In the former, therefore, the mere injection of the bacilli leads to the formation of moderate amounts of the poison, making the difference between experiment and controls a purely quantitative one, whereas in the case of the cocci, no proteotoxin, or practically none, is formed when the bacteria alone are injected. The difference between experiment and control here, therefore, shows much more sharply the effect of the proteotoxin.

In the above experiments we have attributed the increased virulence of the bacteria, when injected together with proteotoxin, to an aggressive property possessed by the poison. This is obviously the only possible interpretation in the case of the staphylococcus and prodigiosus experiments in which the bacteria used in the production of the proteotoxin were of a species other than those later added to the poison and leading to death. In the first experiments, however, in which typhoid bacilli were used, both in the poison production and later in the experiment, another possible contributing factor must be considered. In our earliest accidental observations on the fact that animals would often die of typhoid infection after receiving only the minute doses represented by the bacteria not removable by centrifugation, it had occurred to us that these microorganisms might have been enhanced in virulence because of contact with the serum during the period of incubation incidental to proteotoxin production. This is the more obvious in the light of the recent discovery by Jobling and Petersen (2) that, during such

contact, bacteria will absorb anti-enzymes out of the serum. It might also be correlated with the observation, made by us in isolated cases, that the bacteria cultivated directly from the dead animal showed demonstrably increased resistance to agglutination. However, this latter point is being subjected to further investigation.

Accordingly we performed experiments to determine whether or not typhoid bacilli would increase in virulence after exposure to contact with fresh guinea pig serum, as in the production of the poison.

The procedure adopted consisted in preparing an emulsion of typhoid bacilli, dividing it into two parts, adding definite volumes of fresh guinea pig serum to one, and equivalent volumes of salt solution to the other. After exposure of from five to six hours, both portions were centrifugalized until the supernatant fluids were clear, and approximately quantitative, comparative determinations of virulence could be made with the sediments. The experiments were necessarily inexact owing, in the first place, to the bactericidal action of the fresh guinea pig serum and, in the second, to differences in sedimentation during centrifugation of the bacteria in the salt solution and in the serum. Both errors would tend to favor a negative answer to our query. Careful and repeated performance of this experiment, however, has satisfied us that typhoid bacilli exposed to fresh normal guinea pig serum for five to six hours at 37.5° C. do not gain in virulence sufficiently to influence the results of our preceding experiments. This is as far as we have gone in this direction for the present, since further elaboration of the problem was unnecessary for the specific purposes of the work before us.

Our experiments then have thus far shown that sublethal doses of typhoid bacilli, staphylococci, and, in one case, prodigiosus bacilli could be rendered lethal, if proteotoxin (anaphylatoxin) was administered with them. This aggressive action on the part of the proteotoxin was shown to be non-specific, in that when produced with typhoid bacilli, the poison would serve to augment the infectious properties of staphylococci as well as, or even more effectively than it did those of the typhoid bacilli themselves. This effect of the proteotoxin, however, was not noticeable unless considerable

quantities were employed, no results being obtained unless from 1 tc 1.5 cubic centimeters, representing one fourth to one half of a minimal lethal dose, were injected.

II.

It now became of interest to determine, if possible, the mechanism upon which this aggressive action of the proteotoxin depended. It seemed logical first to investigate whether the proteotoxin acted anti-opsonically; that is, whether it hindered the phagocytosis of the bacteria in the presence of normal serum. Accordingly experiments like the following were carried out.

The anaphylatoxin was prepared from typhoid bacilli, as in the other experiments. That used in the following protocol produced severe shock but not death in a guinea pig weighing 160 grams, when 3 c.c. were injected intravenously.

The bacteria employed were staphylococci emulsified in salt solution from a twenty-four-hour agar slant.

The leucocytes used were obtained by aleuronat injection into the peritoneum of a guinea pig. They were washed once only, since complete removal of the serum seemed unnecessary for this experiment.

The following tubes were prepared, incubated for forty minutes, and slides were made for the determination of phagocytosis in the usual manner.

EXPERIMENT I.

1. Fresh guinea pig serum	0.2 c.c.
Leucocytic emulsion	0.3 c.c.
Staphylococcus emulsion	0.2 c.c.
Salt solution	0.8 c.c.
Phagocytic index = 20 +	
2. Fresh guinea pig serum	0.2 c.c.
Leucocytic emulsion	0.3 c.c.
Staphylococcus emulsion	0.2 c.c.
Proteotoxin emulsion	0.8 c.c.
Phagocytic index = 20 +	
3. Leucocytic emulsion	0.3 c.c.
Staphylococcus emulsion	0.2 c.c.
Salt solution	1.0 c.c.
Phagocytic index = 2.1	
4. Leucocytic emulsion	0.3 c.c.
Staphylococcus emulsion	0.2 c.c.
Proteotoxin	1.0 c.c.
Phagocytic index = 12	

It is apparent from this experiment both in tubes 2 and 4 that the presence of the proteotoxin did not hinder opsonic action. Al-

though the staphylococcus opsonins were considerably diminished by absorption with typhoid bacilli it is evident in tube 4, even after subtracting the opsonic action due to traces of serum carried in with the leucocytes, far from hindering phagocytic action, the proteotoxin still carries with it opsonin for the staphylococci, a fact surprising in itself when we consider the absorption of the serum with not inconsiderable quantities of typhoid bacilli and its exposure, during proteotoxin production, to 37.5° C. for five hours.

It is also possible that, although the heat-sensitive opsonic substances may have been completely or almost completely removed by the exposure to 37.5° C. for five hours, the high phagocytic index in tube 4 may be explained by reactivation of a heat-stabile sensitizing element by the small amount of fresh serum adherent to the leucocytes, which were not thoroughly washed in these experiments. We did not further investigate this purely incidental point.

We shall not cite other experiments since those similarly performed all point, in agreement with this one, to the fact that the proteotoxin, as produced by us, does not exert anti-opsonic action.

From the fact thus demonstrated, that the proteotoxin does not hinder the phagocytosis of staphylococci in the presence of normal active serum, we may reasonably conclude that its action is not anti-opsonic. It is also apparent from the preceding protocol that, although there is slight cytotoxic action on the phagocytic cells, a fact which other experiments lead us to suspect, this is not in itself sufficient to be held responsible for the virulence-enhancing influence of the proteotoxin. For the leucocytes still actively ingested the bacteria in spite of contact, for thirty minutes and longer, with proteotoxin in concentration relatively far greater than any that would be likely to occur in the circulation of the infected animal.

These possibilities being excluded, we turned our attention to the leucopenia which is known to ensue upon the injection of poisons of this kind into animals. The experiment that follows is one of several planned to throw light on this point.

EXPERIMENT II.

After it had been determined on a number of animals that a suitably adjusted quantity of proteotoxin, injected into a guinea pig, regularly produces a fall in the total number of leucocytes, varying in degree, speed of onset, and duration

with the severity of the poisoning, two guinea pigs were intravenously injected as follows:

1. Weight 250 gm.
   Staphylococci $\frac{1}{20}$ agar slant.
   Proteotoxin, 2 c.c.
2. Weight 260 gm.
   Staphylococci $\frac{1}{20}$ agar slant.
   Salt solution 2 c.c.

The leucocyte counts of these animals were as follows:

	1.	2.
3 counts during a period of 4 hrs. before injection	7,200	9,500
	5,800	6,500
	4,000	7,000
1 hr. after injection. 3 separate counts	1,000	1,200
	1,400	1,000
	1,600	1,000
3½ hrs. after injection	3,200	6,000
	1,600	5,800
	1,400	5,600
Next morning about 18 hrs. after injection	1,800	16,000
	1,600	24,000
	2,000	32,000
		18,000

The first animal died within twenty-four hours after the injection, and blood cultures taken from the heart's blood before and after death showed numerous staphylococci. The other animal, in which no proteotoxin had been injected with the bacteria, was slightly sick for five or six hours after the injection, but after that it rapidly recovered and remained well.

In order to obtain results as striking as the one presented in the preceding protocol, it is necessary to carry out a considerable number of experiments until the relative doses of proteotoxin and staphylococci are so adjusted that a marked and consistent difference is apparent. For in many cases a too severe dosage with staphylococci, even without the poison, may lead to a considerable prolongation of the stage of preliminary leucocytic depression, as seen in guinea pig 2 after one hour. A too slight dosage with proteotoxin, on the other hand, will result in a temporary leucopenia only and recovery of the animal. Properly adjusted, however, the experiment is instructive in that it shows that the animal receiving the bacteria together with the proteotoxin does not recover from the

preliminary leucopenia which follows the injection, and does not, later, react with the powerful leucocytosis that accompanies recovery in the control animals.

SUMMARY AND CONCLUSIONS.

1. Our experiments show that proteotoxin (anaphylatoxin) produced with typhoid bacilli and active guinea pig serum, by the general method of Friedberger, possesses aggressin-like action in that, when injected together with sublethal doses of bacteria, it renders them lethal.

2. This action is not specific, since even when typhoid bacilli are used in its production, the proteotoxin possesses this property not only for typhoid bacilli, but for staphylococci and prodigiosus bacilli, the only other organisms with which we worked.

3. This action is manifest in the experiment only when quantities of proteotoxin are used which can produce a certain degree of systemic poisoning.

4. It is not due to anti-opsonic action since the phagocytosis of staphylococci in the presence of active serum takes place readily in spite of the presence of relatively considerable amounts of the poison.

5. The aggressin-like action of the proteotoxin is probably due to the leucopenia that it causes. It is not likely that this leucopenia is dependent upon a negative chemotactic action, in the ordinary sense of this term, but it is probably due to the general intoxication of the animal, resulting among other things in the poisoning of those tissue elements which ordinarily react to infection with the mobilization of leucocytes in the circulation.

It has seemed to us that the results of our experiments were of interest also in throwing some light on the general problem of virulence.

The thought naturally suggests itself that Bail's so called aggressins were nothing more or less than proteotoxins, or anaphylatoxin. It has been shown by Friedberger and Nathan (3) that poisons of this description may be formed in the peritoneal cavities of animals, and if we examine the method by which Bail (4) prepared his aggressive exudates, it seems likely that they contained proteotoxins.

Moreover, although Bail's aggressins are described as non-toxic in themselves, this is a discrepancy to which, it seems to us, no great weight need be attached in this connection, since the poisonous properties of the proteotoxins are clearly apparent only when considerable amounts, two to three cubic centimeters, are intravenously injected into young guinea pigs, and below this quantity a threshold is rapidly reached beyond which no visible reaction whatever is achieved. We have not yet, it is true, shown that animals may be immunized against bacterial infection by treatment with the proteotoxins, a result obtained by Bail with his aggressins. But we feel that the mere fact that tolerance to proteotoxin may be established, a phenomenon which has been the subject of an earlier paper, is a step in this direction. Further than this the analogy cannot be carried since we do not know as yet whether the proteotoxin tolerance is specific,—a fact which would be necessary in order to identify completely these substances with the substances studied by Bail. In view, however, of the many objections that have been voiced against this phase of Bail's work, and of the fact that a rapid accumulation of leucocytes is described as characteristic of aggressin immunity, it seems not unlikely that even in this aspect of the problem aggressin and proteotoxin may eventually be identified.

It is probable, also, if we reason logically from the premises which our experiments supply, that the power of invasion of many microörganisms is intimately related to their ability to react with the active blood constituents of the infected animal. This has already been suggested in the work of Embleton and Thiele (5). If, indeed, the condition of bacterial anaphylaxis or hypersusceptibility depends upon the establishment of a balance between the injected bacterial antigen and the blood constituents, such that proteotoxins are formed, a supposition rendered probable by much recent work, then our experiments would tend to show that bacterial anaphylaxis implies, at the same time, a condition of lessened resistance to invasion by the bacteria[1] (6). It may even be that generalized inva-

[1] Professor William H. Welch, of Johns Hopkins University, has told us that several years ago, in carrying out some experiments with peptone injections, the animals that received the peptone seemed thereby to be rendered more susceptible to infections. Considering the demonstrated similarity between peptone poisoning and anaphylactic poisoning his observation would correspond to that made by us. We have so far had no opportunity to carry out experiments with peptone.

sion, after localization, must await the striking of such a balance, an interval which, in the case of diseases like typhoid fever, may form a part of the incubation time. At the same time, in such diseases, the action of the proteotoxin would explain the leucopenia that accompanies the bacterial invasion.

We do not, of course, expect to explain virulence in general by these phenomena. In the case of organisms like the pyogenic cocci, the pneumococcus, and some other bacteria, there is apparently another important element contributed in the resistance of the invader to direct reaction with the active serum constituents. In many of these organisms the slight extent or absence of serum lysis or even bactericidal action would point in this direction, and here the appearance of proteotoxin would be secondary in importance or possibly absent entirely until late in severe infections.

The non-specificity of the aggressive action of proteotoxin goes far toward explaining the increased susceptibility to other, secondary infections apparent during the course of many cases of typhoid fever and in some other diseases. It should also have an important bearing on our understanding of mixed infections.

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A METHOD OF COUNTING THE ACTUAL NUMBER OF PURKINJE CELLS PRESENT IN A GIVEN AREA OF CEREBELLUM, AND ITS APPLICATION IN TEN CLINICAL CASES.*[1]

By FREDERIC WADE HITCHINGS, M.D.

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In making extensive studies of nerve cell changes, it was found that while a qualitative method was of certain value, a quantitative method would be essential in order to obtain the greatest possible accuracy. This led to the formation of an arbitrary classification of nerve cell changes (Crile) and to the method of making differential Purkinje cell counts by means of which cell activity could be reduced to percentages of active, fatigued, and exhausted cells. The application of these differential counts has been of wide range, and the more counts that have been made under many carefully checked and counterchecked conditions, the more confidence has been felt in their relative accuracy.

The problem of the exhausted cell as presented by the differential counts is of considerable interest. If we assume that a destroyed nerve cell is never replaced, the problem becomes one of great practical importance. The body can regenerate many of its component cellular parts, but if it cannot regenerate nerve cells, if it has but one supply for the use of the individual during his life, every step taken to conserve this supply would be a step toward maintaining efficiency and probably toward prolonging life itself.

The arbitrary definition of an exhausted cell is that it is one in which, after loss of practically all the intracellular and intranuclear chromatin granules, the nucleolus itself begins to disintegrate. It

* Received for publication, September 21, 1914.

[1] This method was elaborated in the course of the routine work done in the laboratory of Dr. G. W. Crile. Thanks are due to Dr. Crile, to Dr. J. B. Austin for the differential Purkinje cell count figures, and to Dr. H. C. King, for assistance in making the actual Purkinje cell counts.

is not known absolutely that a cell in such a condition cannot recuperate, but there is little doubt but that this is so. Beginning nucleolar disintegration is the first stage of exhaustion. With increasing disintegration or disappearance of the nucleolus the major structural changes occur, from rupture and disappearance of the nucleus down to rupture and disappearance of the entire cell.

The assumption is that an exhausted cell in any stage of exhaustion is a dying cell which cannot be recuperated. What becomes of the exhausted cells? Many observers have found evidence of the removal of nerve cells through phagocytic action, and the consensus of opinion is that the phagocytes are not only derived from the white blood corpuscles but also from the neuroglia cells themselves.

The object of this research is to endeavor to show, by a method of making actual Purkinje cell counts, that the observed facts of cell disappearance are supported by demonstrable numerical losses of the cells themselves. With certain reservations it would be expected that in cerebellar tissue showing very high percentages of exhausted cells there would be a corresponding actual loss of cells. If extreme nerve cell activation were to cause extreme nerve cell exhaustion and death in a short time, it would not be expected that many of the exhausted cells would be removed, but in a severe chronic activation the processes of exhaustion and removal would have time to go on and evidence of removal would be found.

THE METHOD FOR MAKING ACTUAL PURKINJE CELL COUNTS.

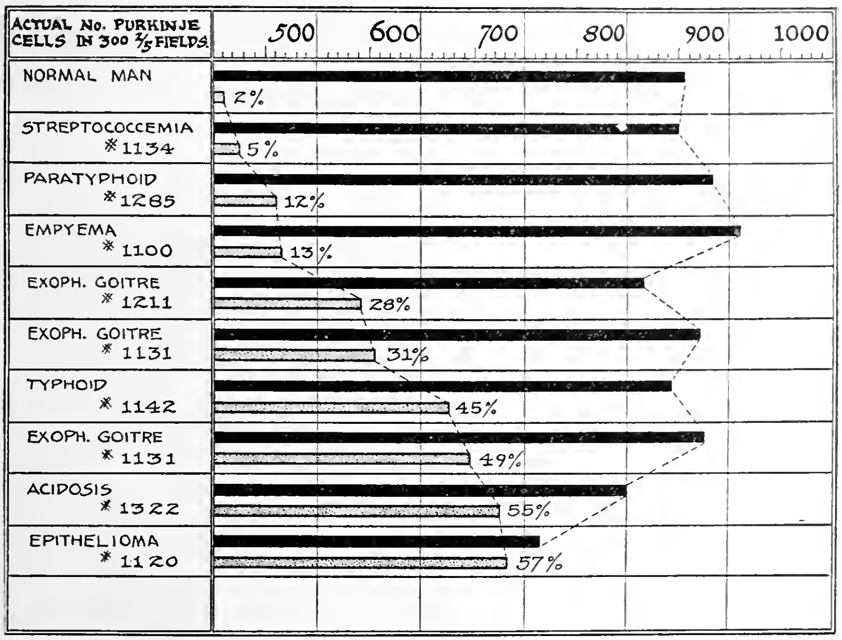
The method consists in counting, with the help of a mechanical stage, the number of Purkinje cells found in 300 fields of the size made by using a No. 2 ocular and No. 5 objective (Zeiss). This can readily be done owing to the linear arrangement of the cells, it being easy to estimate the distance equivalent to the diameter of a field when counting the cells in the angles made by the cerebellar folds. When the cell row is straight it is made to lie in a diameter of the field so as to include as many cells as possible. By following the margins of the cerebellar folds a count begun in one corner of a section can be made to cover the entire section without covering the same area twice. The sections should be of uniform thickness, preferably five microns, and of such a size that an entire count can

be made from one section, or else sections not subjacent should be taken if more than one has to be used.

After counting the cells in the row in a given field the slide is moved exactly one field away, and so on until three hundred fields have been counted, thus covering what is arbitrarily considered to be a large enough area to give a fair idea of the number of cells present, and giving a definite basis for comparison of material from different individuals.

APPLICATION OF THE METHOD.

In applying this method to animals it was found that there was too much variation among normal animals to establish a standard without more labor than could be given at the time (over 100,000 cells were counted). From human brains ten counts were made



TEXT-FIG. 1. The relation between the number of Purkinje cells of all kinds, as shown by actual cell counts, and the number of exhausted Purkinje cells, as shown by differential Purkinje cell counts.

The black lines represent actual counts, while the stippled lines represent the percentages of exhausted cells. Marked actual cell loss is shown only in the cases in which large percentages of exhausted cells were present.

as shown in text-figure 1. The actual Purkinje cell counts are indicated by the solid horizontal lines while the stippled horizontal lines show the percentage of exhausted cells found in the respective differential Purkinje cell counts. The exhausted cell counts are arranged in sequence.

It will be seen that the largest percentage of exhausted cells was in the case of epithelioma and that in this same case the smallest actual number of cells was found (16.6 per cent. less than in the count from the normal man). On the whole, while the curve of actual cell loss does not diminish so evenly nor so markedly as the curve of exhausted cell increase, there is enough convergence to suggest that the underlying principle is correct.

CONCLUSIONS.

1. This method of determining the actual number of Purkinje cells present in a given area of cerebellum is practicable and of sufficient accuracy to make it another useful means of studying nerve cell activity.

2. In its application to clinical cases it is found that increasing nerve cell exhaustion is accompanied by increasing nerve cell disappearance, although it is recognized that theoretically complete nerve cell exhaustion could be present without nerve cell disappearance on account of the individual dying before phagocytic action could take place.

3. This disappearance of nerve cells corroborates the theories and observations made on phagocytosis of nerve cells, inasmuch as it shows that nerve cells disappear from the brain.

4. While there are too few cases to establish a normal actual Purkinje cell count, it is of interest to note that there were 16.6 per cent. fewer cells in the case with the maximum cell exhaustion (57 per cent.) than in the case of the normal man (2 per cent.).

AGGLUTINATION PHENOMENA IN LOBAR PNEUMONIA.*

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Numerous investigators (1 to 11) have noted that when pneumococci are grown in immune serum the organisms exhibit unusual characteristics. Certain observers have also shown that the sera of animals immunized to pneumococci are able to cause agglutination of these organisms when the ordinary agglutination technique is employed.

In 1900 Besançon and Griffon (12) noted the presence of agglutinins in the blood of patients ill of and convalescent from lobar pneumonia. They found that often the homologous strain (the strain derived from the patient) was agglutinated when a heterologous strain was not. This fact suggested that the various strains of pneumococci might possess biological differences which could not be detected by ordinary cultural methods. Eyre and Washbourn (13) and Gargáno and Fattori (14) also noted such differences, but Neufeld and Händel (15) were the first to study thoroughly immunological variations in pneumococci. They worked, however, with relatively few strains of pneumococci.

Dochez (16) and Dochez and Gillespie (17), working with a large number of strains of pneumococci, isolated from patients suffering from lobar pneumonia, were able to differentiate pneumococci into four groups on the basis of cultural and immunological reactions. The three main groups were distinguishable by agglutination and animal protection experiments with a fourth group which includes all strains of pneumococci which cannot be otherwise classified.

More recently Lister (18) in South Africa, approaching the problem from another standpoint, has differentiated into four groups eighteen of twenty strains of pneumococci isolated by puncture of the lungs of patients having lobar pneumonia. This he was able to do by cross agglutination tests with cultures of these strains of pneumococci and the sera derived from these patients at about the time of crisis. Two strains he found to be non-agglutinable.

The present study has been carried out with the purpose of determining the time of appearance and disappearance of agglutinins in the blood of patients suffering from pneumonia, and of learning more concerning the specific character of these agglutinins, especially their specific relation to the various groups of pneumococci.

* Received for publication, October 3, 1914.

METHODS.

Neufeld (19), Jehle (20), Kindborg (21), and others have found that pneumococci are agglutinated by the serum of immunized animals when this is diluted to 1 to 60 or even 1 to 100,000 (21), and that the serum of patients diluted even to 1 to 160 may cause such agglutination when the homologous strains are used. We have found, however, that the reaction is highly specific even as regards the various groups of organisms, so that for the purpose in view the use of diluted serum seemed unnecessary. This is especially true since normal human blood does not contain agglutinins for the pneumococcus, and spontaneous agglutination of pneumococci does not occur with the method employed. Therefore, in making these tests equal parts (0.3 of a cubic centimeter) of a broth culture of the organism to be tested and of the patient's serum were mixed together and macroscopic readings were made after two hours in a water bath at 37° C. and after twenty-four hours in the ice-box. In practically every case agglutination was apparent at the end of the two hour period, if it occurred at all.

The cultures of pneumococci were obtained from culture of the heart's blood of a mouse inoculated intraperitoneally with the patient's washed sputum, or from direct blood culture, or from culture of material obtained by puncture of the lung of the patient.

Certain observers (22) have noted that cultures fresh from the human host are occasionally not agglutinable. Therefore, all cultures were grown for several generations on artificial culture media. To make sure that the cultures employed were agglutinable, they were all tested with antipneumococcus serum specific for groups I and II, before they were used for the agglutination tests with human serum. At each examination the patient's serum was tested against the homologous organism and also against stock cultures belonging to groups I and II.

The blood to be tested was drawn aseptically from an arm vein of the patient. It was allowed to clot, was centrifuged, and the clear serum was pipetted off. The tests were made with serum as fresh as possible, for it has been found that agglutinins in human blood disappear quickly after it is stored. This is especially so if the serum has not been separated from the clot. With highly im-

mune horse serum this is not the case. Serum two years old has been found still to possess strong agglutinative properties.

The correlation between the clinical severity of lobar pneumonia and the bacteriological findings has not only prognostic importance, but also considerable bearing on the effects of serum therapy. It has been learned that infections which are included in groups I and II are usually severe. The serum treatment of lobar pneumonia has been more successful in the cases included in group I than in group II, and experimentally it has been found that antipneumococcus serum I (a serum produced by highly immunizing a horse to pneumococci belonging to group I) possesses greater protective and curative properties than a serum similarly produced by organisms included in group II. It will be noted in the protocol that six of the fatal cases were group II infections, some of which were thoroughly treated with serum.

On the other hand, infections due to organisms in the heterogeneous group (group IV) are almost always mild and the prognosis is favorable. Infections due to *Pneumococcus mucosus* (group III) are often severe, but as this organism under ordinary cultural methods is non-agglutinable, this group may be left out of consideration for the present.

Bearing in mind these apparent differences in the type of disease produced by the different groups of pneumococci, we may make an analysis of the results of the agglutination reactions.

DISCUSSION.

The protocol of all the cases studied is briefly summarized in table I.

TABLE I.

Group.	No. of cases.	Agglutinins present at some time in the disease.	Never present.	Per cent. present.
I	16	16	0	100
II	13	7	6	53.8
III (*mucosus*)	2	0	2	0
IV (heterologous)......	9	5	4	55.5

In all, sera from forty cases of lobar pneumonia, due to different types of pneumococcus, were examined. The sera of all the sixteen

cases belonging to group I showed the presence of agglutinins at some stage of the disease. Six of the thirteen cases in group II showed no agglutinins. In five of these negative cases the disease terminated fatally. Repeated examinations of the sera of these patients showed no agglutinins for their own organism or for stock cultures I and II. In all these cases there was no doubt as to the type of the infecting pneumococcus, for four had positive blood cultures and from the fifth an organism belonging to group II was recovered by lung puncture. This confirms the observations of Lister, who was unable to demonstrate the presence of agglutinins in the sera of four cases that terminated fatally.

The impression has been gained that the non-development of agglutinins in the sera of patients with severe infections is evidence of grave prognostic import. Exceptions to this observation have been noted, however, in which a feeble agglutination reaction developed late in two fatal cases. In one of these the patient's serum showed agglutinins on the thirteenth day (the day of admission to the hospital), the patient dying of a complicating meningitis and empyema on the seventeenth day. This case had no serum treatment. In the second case, which was treated with antipneumococcus serum, agglutinins were demonstrable on the thirteenth day of the disease. This patient had four lobes involved and died in extreme dyspnea on the following day.

Observations were made on only two patients infected with *Pneumococcus mucosus*. Hanes (23) has shown that members of this group are specifically agglutinable when treated according to the method of Porges (24), but that they do not agglutinate when subjected to the usual agglutination method. Since the latter method was employed in the present investigation, no agglutinins were demonstrable in the cases recorded.

In four of the nine cases belonging to group IV, no agglutinins were noted in the patients' sera. Two of these negative cases were mild in type, one was quite ill, having a positive blood culture, and the fourth was complicated with influenza. From this last case large numbers of *Bacillus influenzæ* were recovered from the sputum. Unfortunately a lung puncture yielded no culture. It is impossible to say absolutely whether *Bacillus influenzæ* or an atypical pneumo-

coccus or both were the etiological factors. In four of the five cases showing agglutination of the homologous organism, the reaction was feeble and lasted only one day, and in one case, which presented a very severe clinical picture, with positive blood cultures and spreading lesions, the serum developed strong agglutinative power for the patient's own organism on the day of the crisis (ninth day), which power persisted for eighty-four days. This patient later developed an abscess of the opposite lung and resolution of the original lesion was very slow. As the agglutinative power of this patient's serum was so strong and persistent, a few attempts were made to agglutinate with this serum other strains of organisms belonging to group IV, but all with negative results. It has seemed that in this case, as in two others in which agglutinins were demonstrated over a period of 117 days and 112 days respectively, the duration of the lesion (delayed resolution) had some relation to the persistence of agglutinins in the blood. In the other twenty-five cases showing positive agglutination at one time or another, the duration of this phenomenon was relatively short; the longest was twenty-one days, the average about seven days.

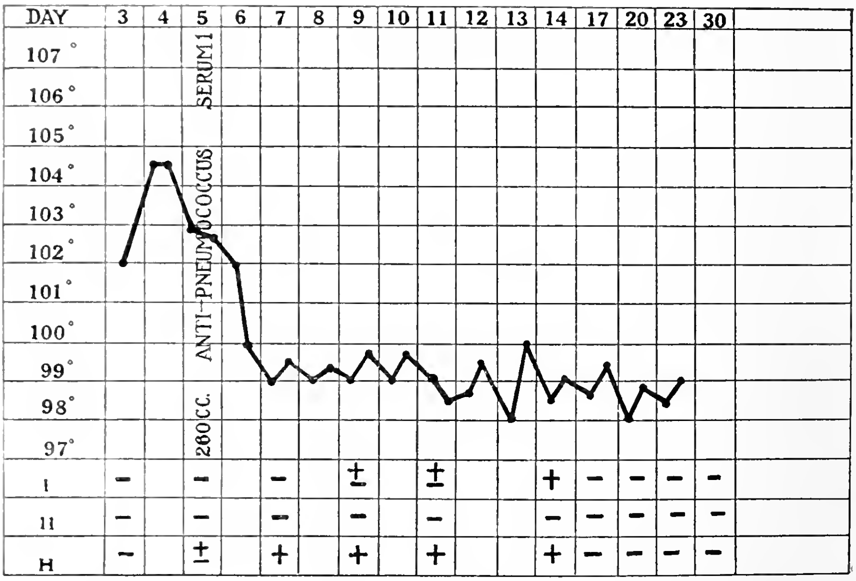
These findings are not wholly in accordance with the results of Jehle's investigations, who found that the sera of only a small number of patients agglutinated the pneumococcus forty-eight hours after the crisis and none after four days.

Agglutinins develop usually just before, at, or just after the crisis (table II, pp. 607–13). During this critical period of the disease Dochez (25) also demonstrated the presence of protective substances in human serum. Only two patients showed agglutinative power any appreciable time before the crisis; one showed it three days before, the second two days before the crisis. The former received antipneumococcus serum, the latter did not.

Too few cases have been studied to draw conclusions as to the effect of serum treatment on the appearance of agglutinins in the patients' blood. Of the sixteen patients in group I, agglutinins appeared in the serum of all. Eleven of these patients were treated with serum, and five received no serum. Of the thirteen cases in group II, agglutinins were demonstrable in the sera of seven cases, and in six cases no agglutinins could be demonstrated at any time.

Eight of the thirteen cases in this group were treated with serum, and of these three developed agglutinins and five did not. On the other hand, four patients showed agglutinins in the blood without serum treatment and none were demonstrated in one fatal case. Thus it would seem that serum treatment had but little influence on the development of agglutinins.

Probably the determination of the presence of agglutinins will have little practical bearing on the diagnosis and treatment of the disease. However, the fact that an organism derived from the



TEXT-FIG. 1. The relative time of development of agglutinins for homologous and stock organisms in a case of lobar pneumonia. I = stock culture of pneumococcus belonging to group I. II = stock culture of pneumococcus belonging to group II. H = homologous culture of pneumococcus, *i. e.*, that derived from the patient.

sputum is agglutinated by the patient's serum is corroborative evidence that this organism is the etiological agent concerned. In practically every case observed, in case the serum agglutinated any organism, it caused agglutination of the organism obtained from culture of the heart's blood of a mouse injected with the patient's washed sputum. Agglutination is of no help in the early recogni-

tion of the type of organism concerned, inasmuch as the appearance of agglutinins occurs relatively late in the disease.

In several instances it has been found that the first organism to be agglutinated was the homologous strain and only later was the stock strain agglutinated. This illustrates the great specificity of the reaction even when no dilutions are used. Clough (26) has noted this marked specificity of the serum of patients recovering from lobar pneumonia in his experiments, demonstrating the power of such sera to render virulent pneumococci phagocytable. One case on which observations were made at frequent intervals shows this group specificity clearly (text-figure 1).

CONCLUSIONS.

1. Agglutinins are present in the blood of patients suffering from lobar pneumonia during some stage of the disease in a large percentage (73.8 per cent.) of the cases due to pneumococci belonging in groups I, II, and IV.

2. In most very severe and fatal cases agglutinins cannot be demonstrated, and it is probable that their absence during the later days of the disease may have unfavorable prognostic significance.

3. No agglutinins are demonstrable by the technique employed in the blood of patients suffering from infection with *Pneumococcus mucosus* (group III).

4. In certain cases agglutinins may be demonstrable for only one day, and in other cases they may persist for several weeks.

5. When agglutinins are demonstrable they usually appear at about the time of the crisis.

6. It has not been possible to demonstrate that treatment with immune serum has any effect on the appearance of agglutinins.

7. The agglutinins present in cases due to organisms of types I and II are always specific for the type of organism causing the infection. In certain cases the agglutination reaction may be more active or appear earlier when the homologous organism is employed than when other organisms of the group are used in the test. In cases due to organisms of type IV, the serum never causes agglutination of any organism except the homologous one.

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TABLE II.

Case No.	Age in yrs.		Day of disease. 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	Days.				Duration of agglutinins in dys.	Type of organism.	Treatment.	Remarks.
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		I																																					−			−	+	+	+				
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1980	7					C																																											
					S	S	S																																									
		I		−		+		+														−																										
		II		−		−		−														−																							2	I	375 c.c. serum I	Recovery.
		H		−		−		−														−																										
1843	35						C																																										

I = stock culture of pneumococcus belonging to group I. II = stock culture of pneumococcus belonging to group II. H = homologous culture of pneumococcus, *i. e.*, that derived from the patient. C = day temperature fell by crisis or lysis. S = days patient received antipneumococcus serum treatment.

TABLE II.—*Continued.*

Case No.	Age in yrs.		Day of disease. 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
					S	S																
		I																		±		
		II																		−		
		H																		±		
1916	8						C															
							S															
		I			−		−		−		±		±			+			−			−
		II			−		−		−		−		−			−			−			−
		H			−		±		+		+		+			+			−			−
2021	30									C												
		I					−			−				+				+			+	
		II					−			−				−				−			−	
		H					−			−				+				+			+	
2012	31					C																
					S	S																
		I		−	−	−					−			±				−				
		II		−	−	−					−			−				−				
		H		−	−	−					+			−				−				
1978	19						C															
					S	S	S															
		I		−	−	−	+							−								
		II		−	−	−	−							−								
		H		−	−	+	+							−								
1939	26						C															
								S	S													
		I				−		−							+							
		II				−		−							−							
		H				−		−							−							
1882	22								C													

Case No.		21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	Days.	Duration of agglutinins in dys.	Type of organism.	Treatment	Remarks
																						17				
	I					−											−					−				
	II					−											−					−	1	I	270 c.c. serum I	Empyema. Recovery.
	H					−											−					−				
1916																										
	I			−							−															
	II			−							−												9	I	260 c.c. serum I	Recovery.
	H			−							−															
2021																										
																						74				
	I																					−				
	II																					−	7	I	0	Recovery.
	H																					−				
2012																										
	I																									
	II																						3	I	360 c.c. serum I	Recovery.
	H																									
1978																										
	I																									
	II																						2	I	510 c.c. serum I	Died. Pulmonary embolism 16th dy.
	H																									
1939																										
																						76				
	I			−																		−				
	II			−																		−	1	I	225 c.c. serum I	Recovery.
	H			−																		−				
1882																										

Case No.	Age in yrs.		Day of disease,																																							Days.	Duration of agglutinins in dys.	Type of organism.	Treatment.	Remarks.
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40					
		I			—			—	—	+			+			—															±																
		II			—			—	—	—			—			—															—													21	I	0	Recovery.
		II			—			—	—	+			+			+															+																
2083	24						C																																								
		I					—		—			—			—			—			—						—							—													
		II					—		—			—			—			—			—						—							—										9	I	0	Recovery.
		II					—		—			+			+			—			±						—							—													
2044	38							C																																							
		I							—			±		+			+					+																									
		II							—			—		—			—					—																						10	I	0	Recovery.
		II							—			—		—			—					—																									
1981	26									C																																					
					S	S																																									
		I		—	—	—			—				—																						±												
		II		—	—	—			—				—																						—									3	I	260 c.c. serum I	Recovery.
		II		—	—	±			+				—																						—												
2008	24						C																																								
											S																															47				
		I								—	—																															—				
		II								—	—																															—	0	II	100 c.c. serum II	Recovery.
		II								—	—																															—				
1880	25											C																																			
					S	S																																					120				
		I								—					—							—																						—				
		II								—					+							±																						—	7	II	175 c.c. serum II	Recovery.
		II								—					+							+																						—				
1827	40					C																																									

TABLE II.—*Continued.*

Case No.	Age in yrs.		Day of disease. 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	Days	Duration of agglutinins in dys.	Type of organism.	Treatment	Remark
		I		—			—		—		—					—					—			—			—															—					
		II		—			—		—		+					—					—			—			±															—		16	II	0	Recovery.
		II		—			—		—		—					—					—			±			—															—					
1953	12						C																																								
																																											116				
										—		—	—		—				—					—		—				—													—				
										—		—	—		—				±					—		±				—													—	8	II	0	Empyema. Recovery.
										—		—	—		—				—					—		±																					
1999	34												C																																		
		I																																													
		II																																										10	II	0	Recovery.
		H												+						+				+																								
1820	31							C																																							
										S	S	S																															122				
		I								—	—	—						—										—															—				
		II								—	—	±						+										+															±	112	II	405 c.c. serum II	Recovery.
		H								—	—	±						+										+															—				
1871	33										C																																				
																																											59				
		I			—		—				—		—			—			—					—			—																—				
		II			—		—				—		+			+			+					+			+																—	20	II	0.	Recovery.
		H			—		+				+		+			+			—					—			—																—				
1969	19								C																																						
		I									—			—																																	
		II									—			—																														0	III	0	Recovery.
		H									—			—																																	
1931	43											Lysis																																			

Case No.	Age in yrs.		Day of disease.																																							Days.					Duration of agglutinins in dys.	Type of organism.	Treatment.	Remarks.
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40									
		I																																																
		II																																																
		H									+											–																												
1876	7									C																																					1	IV	0	Otitis media. Recovery.
											S	S																																						
		I																																																
		II																																																
		H								–	–	–	–	–													–																								
2014	59												C																																				0	IV	185 c.c. serum I	Delayed resolution. Recovery.
																																													46					
		I			–			–						–													–											–							–					
		II			–			–						–													–											–							–					
		H			–			–						+													–											–							–					
1936	22							Lysis																																							1	IV	0	Delayed resolution. Recovery.
		I			–		–			–			–			–			–									–																						
		II			–		–			–			–			–			–									–																						
		H			–		–			–			–			–			–									–																						
2032	20									C																																					0	IV	0	Recovery.
																																											46	63	68	93	100				
		I					–		–		–		–	–					–	–							–					–							–			–	–	–	–	–	–				
		II					–		–		–		–	–					–	–							–					–							–			–	–	–	–	–	–				
		H					–		–		+		+	+					+	+							+					–							±			+	+	+	+	+	–				
2102	18										C																																				84	IV	0	Abscess of lung. Recovery.
		I					–			–	–		–			–																																		
		II					–			–	–		–			–																																		
		H					–			–	–		+			–																																		
2115	12										C																																				1	IV	0	Recovery.

TABLE II.—*Concluded.*

Case No.	Age in yrs.		Day of disease. 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	Duration of agglutinins in dys.	Type of organism.	Treatment.	Remarks.
		I																																									1	IV	0	Recovery.
		II																																												
		H					—						+													—																				
1857	31								C																																					
		I						—		—	—		—		—																												0	IV	0	Recovery.
		II						—		—	—		—		—																															
		H						—		—	—		—		—																															
2093	28														Lysis																															
		I								—			—																														0	IV	0	Recovery.
		II								—			—																																	
		H								—			—																																	
1904	16														Lysis																															
		I													+			+																									3	I	0	Meningitis empyema. Died.
		II													—			—																												
		H													—			—																												
1930	50																																													
						S	S	S	S																																		0	II	1025 c.c. serum II	4 lobes involved. Pericarditis. Died 10th dy.
		I			—		—					—																																	
		II			—		—					—																																	
		H			—		—					—																																	
1839	37																																													
						S	S																																				0	II	375 c.c. serum II	2 lobes involved. Died 5th dy.
		I				—	—																																							
		II				—	—																																							
		H				—	—																																							
1856	50																																													

Case No.	Age in yrs.	Day of disease.																																								Duration of agglutinins in dys.	Type of organism.	Treatment.	Remarks.
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40				
					S	S	S																																						
		I			—	—	—																																						
		II			—	—	—																																			0	II	365 c.c. serum II	Died 6th dy.
		H			—	—	—																																						
1899	62																																												
		I	—		—		—																																						
		II	—		—		—																																			0	II	0	3 lobes involved Died 8th dy.
		H	—		—		—																																						
1983	45																																												
					S	S	S	S																																					
		I		—	—	—	—	—																																					
		II		—	—	—	—	—																																		0	II	1125 c.c. serum II	4 lobes involved. Died 7th dy.
		H		—	—	—	—	—																																					
1835	36																																												
						S	S	S	S	S			S																																
		I					—	—	—	—					—		—																												
		II					—	—	—	—					—		+																									1	II	1175 c.c. serum II	4 lobes involved. Died 17th dy.
		II					—	—	—	—					—		±																												
1858	43																																												
		I			—			—																																					
		II			—			—																																		0	III	0	Died 7th dy.
		II			—			—																																					
1963	57																																												

CULTIVATION OF THE SKIN EPITHELIUM OF THE ADULT FROG, RANA PIPIENS.*

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Plates 32 to 40.

INTRODUCTION.

It has now been definitely shown, as far as connective tissue is concerned, that tissues and cells when isolated from the organism and cultivated in nutritive media grow actively through division. The cultivated fragments of connective tissue grow not only through cell migration, but also in a literal sense; that is to say, the cells increase in number.

Seventy-eight days after the explantation of cultures of rat connective tissue Lambert and Hanes[1] were able to observe microscopically the various stages in the mitotic division of the individual cells, and Carrel[2] has shown that cultivated connective tissue can be preserved in a state of growth for an indefinite period of time.

Recently Carrel[3] has stated that a strain of connective tissue taken from the heart of a chick embryo and cultivated outside the organism for a period of twenty-eight months possesses a much greater rate of activity than do freshly cultivated fragments of connective tissue. Numerous examples of mitosis in these cultures of more than two years' duration demonstrate the active multiplication of the cells.

The conditions governing the cultivation of epithelial tissue, however, are not equally favorable. The behavior of cultivated epithelial cells has been studied mainly in organs of warm-blooded animals containing epithelial cells. As happens in the case of connective tissue, these epithelium cells shortly after they have been placed into the medium begin to leave the tissue and migrate into the medium, thus forming a membrane of cells around the original fragment. Usually, however, these cells soon perish, and it has not yet been definitely ascertained whether or not they possess the power of actively increasing in

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[1] Lambert, R. A., and Hanes, F. M., Beobachtungen an Gewebskulturen in Vitro, *Virchows Arch. f. path. Anat.*, 1913, ccxi, 89.

[2] Carrel, A., Present Condition of a Strain of Connective Tissue Twenty-Eight Months Old, *Jour. Exper. Med.*, 1914, xx, 1.

[3] Carrel, A., *loc. cit.*

number. Lambert and Hanes,[4] who have made comparative studies of cultures of epithelial and connective tissue cells, have stated that epithelium is much more difficult to cultivate than connective tissue. They have apparently seen no cases of cell division, as no mention is made of this phenomenon.

The results obtained with cultures of epithelial cells of cold-blooded animals were even less satisfactory than those of warm-blooded animals. Hitherto these experiments have been performed only with the epithelium of *Anuræ*. The first cultures of frog skin were made by Ruth[5] in Carrel's laboratory. The object was, however, only to investigate certain processes of cicatrization, for which purpose it was not necessary to keep the frog's skin alive longer than three days. During this time the cultures showed a more or less considerable expansion of the epithelial cells which surrounded the old fragment in the form of a compact layer.

Oppel[6] cultivated the entire tail of tadpoles, and although a membrane emanating from the old epithelium was formed to cover the cut surfaces, he was unable to preserve these objects alive for any considerable length of time, and was thus prevented from following the subsequent fate of the epithelial cells.

The reports of Holmes[7] are nevertheless more satisfactory, inasmuch as they are concerned with the potentiality for life of the cultivated epithelial cells. This investigator worked with embryonic frog skin which he cultivated in the plasma or lymph of the adult frog. The epithelial cells separated themselves from the fragments of skin, both in the form of compact membranes and individually. In the latter case they were subsequently able to unite to form secondary epithelial membranes. In the cases of isolated epithelial cells it was shown that the individual cells can be maintained alive as long as two months. There was no positive evidence of cell division.

Connective tissue and epithelium can be clearly distinguished, at least in the early stages, by the shape of their cells and by their respective modes of expansion in the medium; and Lambert and Hanes[8] have recently reëmphasized the fact that they can readily distinguish the two kinds of tissue in cultures. The connective tissue cells never form compact membranes, but yield a kind of network surrounding the old fragment of tissue in which the cells are oblong and spindle-shaped. The exact opposite obtains in the case of cultivated epithelium; its cells are always more or less polygonal in shape and unite to form compact membranes. Added to this is the fact that connective tissue cells can continue to live and grow for years without difficulty, whereas the epithelium cells perish after a short period of membrane formation.

[4] Lambert, R. A., and Hanes, F. M., *loc. cit.*

[5] Ruth, E. S., Cicatrization of Wounds *in Vitro, Jour. Exper. Med.,* 1911, xiii, 422; The Influence of Distilled Water on the Healing of Skin Wounds in the Frog, *ibid.*, p. 559.

[6] Oppel, A., Demonstration der Epithelbewegung im Explantat von Froschlarven, *Anat. Anz.,* 1913, xlv, 173.

[7] Holmes, S. T., Behavior of Ectodermic Epithelium of Tadpoles, when Cultivated in Plasma, *University of California Publications in Zoölogy*. 1913. xi, 155.

[8] Lambert, R. A., and Hanes, F. M., *loc. cit.*

The same morphological differences between the connective and epithelial tissues which exist in the cultures are also found in the organism, in which the two kinds of tissue are strongly differentiated by the above mentioned morphological characters. Whether the same parallel exists between cultivated and normal tissue, as regards the capacity for growth possessed by the two kinds of tissue, and what the causes of this varying capacity for growth are, has not been investigated. This paper is submitted in the hope of contributing in some measure to the solution of this problem.

MATERIAL AND METHOD.

For the purpose of our experiments we selected the leopard frog (*Rana pipiens*), which is easily obtained. We employed as material for cultivation the skin of adult animals, which with the epithelium and cutis was cut into small fragments and placed in the culture medium. The pieces selected for cultivation were usually taken from the back of the frog.

The culture medium was generally composed of a mixture of blood plasma and muscle extract from adult frogs. Great difficulty was experienced from the fact that this mixture coagulates much less easily than some others, than chicken plasma, for example. My observations coincide in many respects with those of Losee and Ebeling[9] in the case of human plasma. The capacity to coagulate possessed by the frog plasma is subject to extreme individual fluctuation, and this is possibly modified further by the seasons. It is probable that this factor is accentuated for a short time following the breeding period.

Semifluid plasma which has not coagulated well is ill suited for the preservation of cultivated tissues for any length of time. For that reason it is of advantage to add a small quantity of chicken plasma to the frog plasma, which effects a satisfactory state of coagulation. Certain plasmas begin by coagulating in a satisfactory manner, but the metabolism of the culture subsequently induces a state of liquefaction. Drops of plasma-muscle extract containing no tissue never liquefy. The same applies to fragments of dead tissue which are only slightly or not at all active. But fragments of skin in full activity give rise within a few hours of explantation to a cavity containing a liquid, even when all the physiological salt solution is carefully removed before placing them in the medium, and they are thoroughly shaken in it before coagulation sets in. The cavity continues to increase the longer the tissue remains in the culture medium, until it attains the dimensions of a drop of liquid. By adding a small amount of chicken plasma to the culture medium the liquefaction can be considerably delayed. The culture medium has to be changed at least every other day, and in cases of liquefaction every day.

[9] Losee, J. R., and Ebeling, A. H., The Cultivation of Human Tissue *in Vitro*, *Jour. Exper. Med.*, 1914, xix, 593.

The cultures of frog skin do not easily support a temperature over 25° C.; temperatures varying between 19° and 21° are more favorable than those above 25° and below 15° C.

EARLY PHASES.

Primary Epithelial Rim.—Soon after explantation changes appear in the cultivated fragment. Figure 1 shows a fragment fixed in formol four hours after removal from the organism and stained by means of Delafield's hematoxylin. Around the fragment of skin, which appears dark in the photograph, a narrow border can be discerned, lighter in color. The latter contains a large quantity of nuclei closely packed together and disposed over each other in several planes. Owing to the density of the tissue the cells belonging to the individual nuclei can be only partially distinguished with the help of considerable magnification, but they are always seen to be polygonal in shape. This primary epithelial layer contains no spindle-shaped cells whatever; the tissue is purely epithelial in character. Certain black cells with branch-like processes which can be distinguished in the deeper strata of the rim of tissue are identical with the small melanophores occurring in the epithelium of frog skin; they have nothing in common with the large melanophores of the cutis and are sharply differentiated from the latter.

Secondary Epithelial Rim.—From sixteen to twenty hours after explantation the cultivated fragments of frog skin are found in a second stage, that of the secondary epithelial rim. This is shown in figure 2. The rim of tissue surrounding the old piece is now seen to have considerably increased in width and its superficial size may even greatly exceed that of the original fragment of tissue. At the same time it has greatly changed in appearance. It now consists of two zones, a narrow central zone and a peripheral zone from five to fifteen times as wide as the former. The central zone represents the upper layers of the primary epithelial rim, which from this time on show no further changes. In the accompanying photograph the central zone appears as a narrow border on three sides of the original piece and is seen to be a little darker than the peripheral zone. As in the case of the primary epithelial rim, this central zone contains small, closely packed nuclei. It is invariably situated above the lighter peripheral zone, so that it looks as if the latter had grown out beyond the central zone.

The peripheral zone is sharply differentiated from the central zone. To begin with, it is lighter and more transparent, owing to the facts (1) that it is composed of fewer layers than the central zone, and (2) that the cells which compose it are flattened out and are hence not so numerous as those constituting the central zone. The character of the tissue is, however, purely epithelial, as appears in figure 2; not a single spindle-shaped cell is to be seen. Nevertheless, the cells composing it are quite different from those of the central zone, as stated above. Each cell can be distinguished individually, and the cell walls are everywhere clearly discernible. The cells are completely flattened, and hence appear large and light in color, and are polygonal in shape. At first they continue to increase in size, as can be ascertained by subjecting the culture to continual observation. The nuclei are large and take the stain very well. They possess one to three nucleoli. Towards the center the peripheral zone consists of

several layers, but at the periphery usually of one only. Immediately behind the peripheral edge a ridge-like zone is often seen, consisting of several layers, the cells of which are closely pressed together. The secondary epithelial rim sometimes contains a very large number of small melanophores, thus showing its epithelial origin. As in the case of the primary epithelial rim, the secondary rim is also nearly uniform; its outer edge is always seen to possess a more or less round, but slightly irregular outline (figure 2).

Loosening Up.—We have seen that during the stages of the primary and secondary epithelial rims the edges of the tissue bordering on the medium are only slightly branched. Everywhere the tissue has the form of a completely compact, continuous membrane. Gradually, however, this picture changes and a progressive loosening up becomes noticeable. Figure 3 shows a culture of another stage to be described later, but it serves to illustrate this process of loosening up. As this process follows the same course in both stages we shall describe it as an example.

First of all, a number of the peripheral cells assume a long, almost spindle-shaped form, as shown in figure 3. Many of them send out long plasma processes into the medium, and these pseudopodia-like processes thus constitute the only link between the cells and the compact parts of the edge of the tissue. They usually assume the form of long chains of cells projecting into the medium, and consist of long, spindle-shaped cells. In this way the line of demarcation of the tissue rim by degrees becomes more and more complicated, and at many places it projects in the form of sharp, jagged protuberances (figure 3).

At the same time spaces are seen to occur in the epithelial membrane, often bridged over by columns of cells. The bridges are also entirely composed of oblong cells, often spindle-shaped. Figure 4 shows a strongly magnified section of the culture seen in figure 3. Most of the cells are still polygonal in shape and their arrangement points to their being strictly epithelial in character. At only one spot where the loosening up process has started can a space be seen in the epithelial membrane, and this is bridged over by a chain of elongated cells.

When the liquefaction of the plasma is very great the loosening up stage assumes a somewhat different aspect. Figure 5 shows a culture which underwent this stage with a rather liquefied plasma. Certain sections, especially those on the right and left sides, where the plasma has remained fairly solid, are seen to have remained compact and to have a very marked fringed edge. In places where great liquefaction has occurred, the membrane has become torn, consequent upon the surface tension of the drop. Large spaces have been formed, partially bridged over by the columns of cells. The cells, following the greatest tension, range themselves at the edge of the drop in the form of a wreath of cells, the columns break down, and their central parts withdraw to the edge of the piece of membrane. If the liquefaction is still more extensive the entire rim of epithelium is torn away from the fragment and a wide wreath of cells is formed, completely surrounding the entire piece of membrane. This wreath at first consists of polygonal and spindle-shaped cells and is connected with the piece of membrane by means of the columns of cells (compare figure 6, slightly magnified, and figure 7, more highly magnified). Later all the columns are broken down by the action of the cells, now spherical in shape (figure 5,

above, central part of the column), which withdraw from the middle of the column towards the periphery and center. The cells constituting the wreath likewise soon become spherical in shape, and the less favorable the conditions of nutrition the more rapidly is this change accomplished, and by the greater number of cells.

Resting Stage.—If the fragment of membrane, together with the surrounding tissue, can be successfully transferred into the fresh plasma, it becomes possible to prolong somewhat the period of the protrusion of chains of cells into the medium. Usually, however, as a result of the liquefaction of the plasma only a narrow rim of cells can be transferred with it into the medium, and owing to the continued loosening up and separation of the individual cells which actively traverse wide portions of the medium, this eventually disappears altogether. Thereafter no additional cells advance from the original culture, and after about seventy-two hours a period of complete rest sets in. From this time on no further changes which might be referred to activity are to be observed, even in portions of the epithelial ring which may yet exist. Only single cells become separated and stray out into the medium; the remainder are still merely subject to change of form which can be attributed to the effect of metabolism. This we shall discuss elsewhere.

Secondary Activity.—The resting stage usually lasts three to four days; namely, from the second to the sixth day after explantation. In all cultures which have not perished through disturbances of nutrition this interval of several days is followed by a period of renewed activity, and the culture passes into the stage of secondary activity. Figure 5 shows a culture during this stage. The culture was made on June 4, and twenty-four hours later had reached the stage of the secondary epithelial rim. Twenty-four hours later, on the morning of June 6, the dissolution was already very much advanced. When the medium was changed almost the entire epithelial rim became dissolved. The following days showed no changes, although the entire piece of skin, as well as the remaining small fragments of the epithelial rim, appeared to be alive and healthy. On the morning of June 8 the medium was again changed, and on the morning of June 9 hardly anything remained of the secondary epithelial rim. On the same day the medium was changed for the third time and shortly afterwards a new rim of epithelium began to develop around the old piece of skin. Numerous identical instances were observed, but we shall confine ourselves to a report of one example.

The tertiary epithelial rim produced by the secondary activity differs but slightly from the first formed secondary epithelial rim. The central zone, corresponding to the primary epithelial rim, which was not concerned in the various changes which have been described, still exists in many cases. It can be made out fairly well in figure 5 as a grey edge surrounding the object. The peripheral zone of the tertiary rim of epithelium is sometimes thinner than that of the secondary rim, and the cells are polygonal in shape and often exceedingly delicate. The small melanophores are either entirely absent or present only in small numbers.

Figure 5 shows furthermore that the tertiary rim of epithelium is subject to the same fate as the secondary. The loosening up of the tissue, the formation of columns and wreaths of cells, and the straying of the cells into the

medium are phenomena which arise as early as twenty-four hours after the first appearance of the tertiary rim of epithelium and lead to a complete breaking up of the tissue membrane.

The behavior of the cultures described above may be considered as typical of the cultures of skin from the back of the adult leopard frog, since these five successive characteristic stages can be observed in most cultures. At the same time it represents the best result that we have thus far been able to obtain with our method. Certain other cultures, however, showed a somewhat different behavior, but it was not always possible to determine the reason of this. Of these exceptions we shall mention one.

In some instances the fragments of skin, after being transferred into the culture medium, remain completely inactive. The first epithelial rim alone is formed and is often extremely narrow. The cultures remain in this condition for some time, although they are treated in exactly the same manner, as far as this can be controlled, as the rest of the active cultures. In the case of culture A 430, for instance, beyond the formation during the first few hours of the first primary epithelial rim not the slightest change appeared in five days, although during this period it was twice supplied with fresh medium. Not until the fifth day, after the third change of medium, did the culture enter upon its active stage. Figure 3 shows this culture on the morning of the sixth day after explantation. By this time the secondary epithelial rim had already reached the loosening up stage (see also figure 4, which represents a fragment of culture A 430 a, highly magnified). The epithelial membrane which, for reasons to be explained later on, consisted only of basal epithelial layers and contained no small melanophores, was extremely thin from the outset. But later, when the loosening up set in and the cells began to project in long strings into the medium it became even more delicate. This can be seen from a comparison of figures 3 and 8; the latter represents the same culture as figure 3, only one day later; *i. e.,* the seventh day after explantation. Moreover, this figure clearly shows the gradual transformation of the polygonal epithelial cells into cells of the spindle-shaped type. Further towards the center where the cells are still united to form a compact membrane, we see everywhere these polygonal forms. Towards the

edge where the cells project into the medium the spindle-shaped type is seen to preponderate.

It should be remarked, with respect to the subsequent fate of culture A 430 a, and of similar cultures which are late in becoming active, that a short time after the period represented in figure 8 the culture had been provided with fresh medium for the fifth time. As a result the entire secondary epithelial rim disappeared. Nevertheless, on the following day, the eighth day after explantation, an equally large rim of epithelium had arisen, which could only be distinguished from the first by its much greater delicacy.

In our discussion of the regular cultures we had reached the fifth stage, that of the secondary activity, and in connection therewith we remarked that the tertiary epithelial rim formed during this stage had suffered dissolution, as in the case of the secondary rim. The original fragment of skin, that is, all which still remained of it, was nevertheless by no means destroyed; on the contrary a new, characteristic stage had developed, the cystic stage. Before discussing this it is necessary to make brief mention of the remarkable change of form undergone by the epithelial cells.

EPITHELIUM AND CONNECTIVE TISSUE.

The tissues of organs consisting of epithelium and connective tissue freshly removed from the body treated by the usual histological methods are seen to be sharply differentiated. Lambert and Hanes[10] emphasize that the same is true of fragments of organs of warm-blooded animals, when cultivated in nutritive media. The epithelial cells are invariably cuboidal, cylindrical, flat, or polygonal in shape, while the connective tissue cells are long and more or less spindle-shaped. Thus, the term "spindle-shaped epithelial cells," as here used, must sound anomalous; and if we furthermore refer to figure 9 as illustrating such spindle-shaped epithelial cells, doubt may arise as to whether the cells are not in reality connective tissue cells. In order to remove this doubt we shall submit some of the facts which serve to prove the epithelial origin of the spindle-shaped cells in the cultures of frog skin.

The fact that no mention has been made of changes occurring

[10] Lambert, R. A., and Hanes, F. M., *loc. cit.*

in the connective tissue, which, of course, was explanted with the epithelium of the skin, may appear remarkable. In none of the skin cultures did the connective tissue of the cutis show the least trace of activity. As the relation between connective tissue and epithelium is in great contrast with the facts ascertained from observation on warm-blooded animals, we have devoted much attention to this point. From the outset the explanted fragments of skin were continually observed, drawn, photographed, and stained. Nowhere could any spindle-shaped cells be detected in the act of separating from the culture. If figure 2, for example, be studied it will be seen that no cells can be discovered bearing any resemblance to connective tissue cells. Many cultures were fixed and stained; in this state they could be viewed from above and below, the magnification could be increased or diminished, but no connective tissue could be discovered. Numerous cultures were examined by this method with unvarying results, as was also the case when they were examined during the earlier stages. Figure 1, representing culture A 429, which had been fixed and stained three hours after explantation, illustrates an early stage. The primary epithelial rim surrounding the fragment of skin shows unmistakable epithelial characters; no spindle-shaped cells can be detected.

The proof of the fact that the membranes composed of polygonal cells surrounding the fragments of skin are in no way connected with the connective tissue lies in the presence in the membranes of small melanophores, which of course are found only in the epithelium and are sharply differentiated from the large melanophores of the cutis.

The continual straying away of the epithelial cells in such large numbers leads finally to the cutis becoming partially freed of epithelium. In this condition the cells can be clearly distinguished in the remaining part, which is composed of connective tissue and is often completely devoid of epithelium; and it can be clearly seen that the connective tissue is definitely and completely circumscribed, and not a single one of its cells has encroached into the surrounding epithelial membrane. So sharply defined is the separation of the connective tissue from the surrounding parts that even the fragment of cutis shrinks into the form of a spherical disc (figure 8).

These facts suffice for the present to prove the epithelial nature of the spindle-shaped cells of cultures of frog skin; later this question will be discussed more fully and further facts will be added.

If the cells shown in figure 9 are epithelial cells, the question arises whether we are further justified in considering the varying form of the cells as a distinct characteristic, which could serve to distinguish connective tissue from epithelium. In the organism another characteristic appears as peculiar to the cell of the connective tissue; namely, the capacity to form fibrillæ. In the tissue cultures, however, in which the connective tissue cells do not always form fibrillæ, we must depend chiefly upon the differences in form of the individual cell species, which fail absolutely in cultures of frog skin. The latter even prove the incontestable fact that the epithelial cells under the special conditions obtaining in the cultures are able to assume the exact form of connective tissue cells. Therefore we must accept the fact that the form of the cells, at least in the tissue cultures, can in no wise be taken to determine whether the origin of a cell is connective tissue or epithelium.

This recalls the reports of cases in which in cultures of organs containing both varieties of cells, *i. e.*, connective tissue and epithelium, the epithelium soon became supplanted by connective tissue, so that ultimately only connective tissue cells remained. The curious changes in the form of the epithelial cells of the frog above described are thus seen to assume an entirely different significance; and it would be a matter of great interest to determine further whether in these instances the epithelial cells are able to assume forms sufficiently distinctive to differentiate them from the connective tissue cells.

DIFFERENCES BETWEEN THE VARIOUS EPITHELIAL LAYERS IN REGARD TO THEIR ACTIVITY.

Not all the layers of the epithelium participate to the same degree in the processes of activity that are observed in a cultivated fragment of skin from the adult frog. The changes occurring in the cultivated fragments of skin depend mainly on the capacity of the epithelial cells to move actively and modify their form. Thus, it is

shown that this capacity is not possessed in the same degree by all the epithelial cells.

The primary epithelial rim (figure 1) needs but a short time for its development. Immediately after explantation into the culture medium, and often while the fragment of skin is yet in the physiological salt solution, this compact rim of epithelium begins to spread over the edges of the fragment. The borders of the primary epithelial rim adjoining the medium are always more or less parallel to the edges of the fragment of skin, by which it is seen that all the cells along one line advance with uniform speed, this being a case of true epithelial movement (Oppel[11]). After a few hours (4 to 24) the epithelial rim becomes more extended; it is still parallel with the cut edges of the epithelial rim, but ceases to be uniform in all the epithelial layers. The cells situated in the deeper layers gradually spread apart to form the peripheral zone of the secondary epithelial rim, whereas the cells of the upper layers completely cease all movement and form the central zone of the secondary epithelial rim. All subsequent changes in the epithelial membrane are confined to the elements of the lower layers, while those of the upper, belonging to the central zone, remain completely inactive and fail to contribute to the formation of the large peripheral zone.

The question now arises: Of what cells are the individual zones composed? This question must be answered if we are to discover what are the specifically active elements of frog skin epithelium. In certain cultures in which the medium was sufficiently firm it was possible to transfer the fragments of skin into the new medium together with the epithelial membrane; but an extremely thin layer became disconnected from the upper surface of the original fragment and was therefore separately placed into a drop of culture medium. A complete, secondary epithelial rim was adherent to the original culture upon transference into the new medium, but the central zone was missing, this having been formed from the separated upper layer. Upon microscopical examination the latter was seen to be composed of several (probably two) layers of large, ex-

[11] Oppel, A., Causal-morphologische Zellenstudien. V. Die aktive Epithelbewegung, ein Faktor beim Gestaltungs- und Erhaltungsgeschehen, *Arch. f. Entwcklngsmechn. d. Organ.*, 1912, xxxv, 371.

tremely transparent, polygonal cells, containing large nuclei and often fine, brownish granules. In figure 10, especially on the left side, these cells can be distinctly seen through the other layers. This membrane was thicker at the edge, at which point the cells were somewhat pressed together. This group of cells corresponded exactly in width to the central zone from which it had been formed at the time of its connection with the culture.

The cell layers concerned in the formation of this upper membrane are obviously the uppermost layers of horny cells of the epithelium. At first the elements constituting them advance into the medium, together with the cells situated beneath them, but this movement soon ceases altogether. If these uppermost layers be isolated from the remainder of the epithelium and maintained in a separate daughter-culture, their cells are seen to possess almost no individual activity at all. They remain alive in the medium for a long time, which fact can be ascertained by means of appropriate nuclear staining. But only the cells situated at the periphery undergo a slight change of form and become somewhat oval. However, this change of shape takes place very slowly. The fact that they nevertheless advance a short distance, in conjunction with the lower layers of the primary epithelial rim, is probably owing to the fact that they are passively carried on by the lower cells; however, as soon as they are disconnected from these cells, all movement ceases.

In many cultures during the stage of the primary epithelial rim a membrane is separated from the upper surface of the culture, which is more compact and consequently darker than the one just mentioned. Upon microscopic examination this membrane was further seen to contain the small melanophores, as shown in figure 11. It consisted of several layers of cells. Figure 10 illustrates the membrane shown in figure 11, seen from below and magnified 370 times. It consists, first, of several layers of small, polygonal cells, among which the small melanophores are distributed, and, second, of the large, flat cells of the uppermost layer mentioned above, which are seen through the layer of melanophores when properly focussed. Thus, while the first mentioned membrane contains only the upper layers, we here have before us the upper and middle epithelial layers, the latter together with the small melanophores. A layer consisting

of such cells could only be separated from cultures which had not yet formed a secondary epithelial rim; but the latter often arose after these layers of skin had become separated, which proves that the basal cells of the epithelium had remained attached to the fragment of skin. However, when the secondary epithelial rim had been formed only after the separation of the upper and middle layers, it naturally had no central zone and was always devoid of small melanophores, as can be seen in figures 3 and 8. But the cells composing it were always typically epithelial.

The membrane of cells consisting of the upper and middle layers of epithelium was separated from the original culture by placing it into separate ones, after which it was maintained in a special daughter-medium. Figure 11 shows one of these membranes three days after isolation. Only a few cells (from the upper edge) are seen to have strayed into the medium, but slight traces of activity could be discerned as late as eight and nine days afterwards. This layer also, when separated from the fragment, was seen to be almost inactive.

It now remains to be shown what attitude is adopted by the basal cells after they have been isolated. To begin with, it could be seen, from the numerous cases in which the secondary epithelial rim was formed only after the separation of the upper and middle layers, that these cells are able to advance actively, even without the cooperation of the upper layers of cells. If a fairly large section be isolated from such a rim of cells and be placed separately in a drop of culture medium, the cells are seen to behave exactly as though they were connected with the whole epithelial rim; they lose their polygonal form and wander out into the medium under the most varied forms, by means of which the entire complex of cells becomes dissolved.

Thus, in the explanted frog skin we are able to distinguish three layers of epithelium physiologically of different value: (1) A basal layer, the cells of which are set in great activity through the conditions prevailing in the cultures; they maintain this activity even when they are kept completely isolated. (2) A middle layer containing the small melanophores, which certainly participates, at least as far as the latter are concerned, in the formation of the

secondary epithelial rim. When, however, it is isolated with the third layer its cells have hardly any power of movement and can advance only slightly into the medium. (3) An uppermost layer whose capacity for expansion is confined to the formation of a narrow rim and disappears altogether upon isolation.

It was not possible at this stage to ascertain whether this physiological differentiation into three layers of the epithelium of the frog's skin coincided with the morphological differentiation, which similarly shows three layers. It is certain, however, that the differences between the individual layers of shifting epithelium are of great significance for a number of normal and pathological morphogenetic processes, for which reason we desire to give special attention to this fact in our subsequent investigations.

Thus, the formation of the secondary epithelial rim is mainly attributable to the activity of a basal layer of cells; at first these cells move as compact membranes into the medium, later on they become separated, the epithelial cells which were hitherto polygonal in shape become spindle-shaped, and after having become isolated they move like connective tissue cells.

From this we see that the epithelial cells are by no means compelled to assume the epithelial movement which Oppel has designated as characterizing the epithelium; this occurs only as long as they form compact membranes. If they become very much separated from each other the manner of their movement is the same as that of connective tissue cells. Not alone with regard to their form, but also through their manner of movement the epithelial cells are capable under special circumstances of approaching the type of the connective tissue cells. It is interesting to note in this connection that Holmes[12] saw a movement of the epithelial cells caused by pseudopodia in the explanted skin of young larvæ of *Anuræ*.

THE EPITHELIAL CYST.

It has been shown that in the explanted fragment of skin stages of activity alternate with stages of rest. Thus far it has not been possible either to maintain the cultures in continuous activity or to induce a state of activity more than twice. In the active stage a

[12] Holmes, S. T., *loc. cit.*

rim of epithelium of a relatively large area is developed each time around the fragment, after which exhaustion occurs. But the fragment of skin by no means dies; on the contrary it undergoes a series of extremely interesting changes, which bear witness to its undiminished vitality. Even as early as the stage of secondary activity, *i. e.*, from the sixth to the seventh day, the fragment of skin, which was originally square, becomes drawn into the form of a spherical disc whose edges tend to bend down. This contraction of the tissue continues until we finally see it in the shape of a sphere. At first it is pigmented only on the upper surface, while the under surface is whitish in color and resembles closely an amphibian egg. By degrees, however, the pigment spreads to the under surface until finally the whole sphere is brownish in color.

The uppermost layer is usually discarded a few days after explantation, and frequently the middle and upper layers of the epithelium have also become separated; but in spite of this fact a loose clinging layer is soon observed around the sphere, consisting of more or less polygonal and usually rather oblong cells. This layer becomes detached in the course of one of the transplantations into the new medium, whereupon a new one is immediately formed, which may also become detached. In this manner a fragment of skin discards three layers of cells in the course of twenty days, besides which twice during the active stage it sent a considerable number of epithelial cells into the medium. Figure 12 represents one of these cysts, aged eighteen days, and likewise shows a layer in the act of becoming detached, which can be seen particularly clearly on the lower surface.

The cell membranes which have been discarded during the cyst stage consist in part of dead, nucleus-free cells, and in part of living cells of the same form as those constituting the uppermost epithelial layers, and, like the latter, contain in their plasma fine, brownish granules. The nuclei are easily stained. If the first epithelial layer consists of horny cells, as is generally assumed, this can also, on account of the absolute identity of the cells, be taken as being the layer of the cells which have been discarded during the cyst stage. Thus, we are here concerned with a process of shedding.

In spite of the repeated and extensive loss of epithelium, the sphere, which is filled with well preserved connective tissue (as can be seen in sections), still possesses a considerable covering of epithelium. This is seen to be differentiated into separate layers of cells. The innermost layer consists of cuboidal cells, which supposedly correspond to the basal cells. Next to these is a layer which usually consists of two rows of cells which are flattened, and the nuclei of which together with the long axis are parallel to the upper surface. The nuclei of both layers stain well. At the exterior is another peel-like layer of extremely flattened cells, which are separated from the two inner layers of cells by means of a small space, the cells of which are only loosely connected. Small melanophores are found in none of the three layers of cells.

From the sections it can be seen that not only the epithelium, but also the stratum spongiosum of the cutis has completely enveloped the stratum compactum and has assumed a spherical form. This explains the complete pigmentation of the cyst.

We may now inquire whence is derived the inexhaustible supply of epithelial cells. Unfortunately, it has not been possible thus far to answer this question. In the first place, it is inconceivable that the original epithelium could repeatedly give off such relatively large masses of cells without undergoing multiplication; but, on the other hand, we nowhere see any trace of mitosis. In the cysts the epithelium shows a distinct differentiation into a basal layer of cubic cells and into a second layer lying above it, consisting of several layers of flat cells. But the basal cells are nowhere found in a state of mitosis.

Thus, for the present, we must content ourselves with establishing the fact that the explanted fragments of skin are gradually transformed into a spherical form, completely shut off towards the exterior, containing a compact connective tissue core and an epithelial coating. In this form the cultures of skin remain alive for a long time and even show the process of shedding which characterizes normal epithelium.

MECHANISM OF THE EPITHELIAL GROWTH.

The life cycle of a culture of skin may serve to throw some light on the mechanism of epithelial growth.

As mentioned above, it is usually much easier to cultivate connective tissue than epithelium in culture media. The conditions created through life *in vitro* are much more favorable for connective tissue than for epithelium, for the reason that explantation modifies to a much less extent the normal manner of growth of the former than of the latter.

In cover-glass cultures the tissues are surrounded by the medium equally on all sides; this practically corresponds to the conditions under which connective tissue lives in the organism under normal conditions, where it is always surrounded by the body juices.

Craster[13] has proved in the case of the epithelium of rat skin that through subcutaneous transplantation of fragments of skin the conditions become so changed, owing to their transference to the interior of the body, that in a very short time the epithelium entirely lacks the power of resuming its normal function when transferred back to its original position. In cover-glass cultures the fragments of skin are subjected to similar abnormal conditions, for they are excluded on all sides from the external world by means of the medium. But it is a characteristic of all epithelia that they possess not only a basal, but also a free surface; they are nourished from the former exclusively, whilst the free surface is differentiated to fulfill special functions. Thus, all epithelial cells, as well as the entire epithelium, are always differentiated as a whole along a polar axis which is perpendicular to the surface of the organ. The connective tissue cells grow freely in the organism in all directions, whilst the epithelial cells under normal conditions grow almost exclusively in a vertical direction; *i. e.,* along an axis perpendicular to the surface of the organ. Under abnormal conditions, as in the case of regeneration, this growth is parallel to the surface of the organ; the cells, however, never grow freely, but along the surface of the organ.

While the connective tissue is able to develop nearly all its characteristics normally in the medium, the explanted skin epithelium is confronted by entirely different conditions. The isolation of the fragment of skin and the consequent formation of free wound surfaces immediately give rise to regeneration, as would also have

[13] Craster, C. V., Conditions Governing the Growth of Displaced Normal Tissue. *Jour. Exper. Med.,* 1912, xvi, 493.

occurred in the organism. The enormous expansion of the epithelial cells into a surface parallel to the upper surface of the fragment of skin corresponds in every particular to the behavior of the skin epithelium in the organism when stimulated by incisive wounds. However, it continues to spread along the surface of the organ deprived of epithelium and the epithelial cells thus suffer either no suspension at all of their polar differentiation or only a passing suspension. But in the plasma cultures the epithelial cells stray freely into the medium, thus losing ground beneath them, and by this means they enter upon physical conditions similar to those of normal connective tissue. They therefore abandon their polar differentiation and take on the morphological character also of connective tissue cells. Their compact epithelial arrangement vanishes and the cells become spindle-shaped. Not meeting with any impediments tending to terminate their movement as under normal conditions, they continue to wander away from the explanted fragment of skin, and the epithelial rims are finally reduced to complete dissolution.

This never ending spreading out of cells is not terminated in the cultures by means of contact stimulus, as happens under normal conditions. Thus, in spite of the fact that a considerable number of living epithelial cells remain, the activity of the fragment of skin becomes exhausted, and the formation of epithelial rims which glide out into the medium becomes suspended. Meanwhile, an increasing contraction of the explanted fragment of skin has already taken place, and the latter soon becomes spherical in form. Fragments of skin which were examined during this stage were already found to be completely surrounded by epithelium. But the fact is by no means excluded that this surrounding of the fragment of skin was initiated immediately after explantation, and that it is owing to this that the connective tissue cells are prevented from straying into the medium and that the contraction of the fragments of skin is brought about. We shall later on institute more detailed investigations with regard to this point.

The epithelium is thus brought into a condition much more nearly approaching normal. The growth which at first was parallel to the upper surface naturally ceases the moment that the edges of the epithelium, which on both sides are growing around the connective

tissue core, come in contact with each other, thus forming a hollow sphere. The conditions prevailing on the inner and outer sides, respectively, of the epithelium are now different, as happens also under normal conditions. Although the outer surface is not in contact with water or damp air, as would be normal in the case of frog skin epithelium, there are present nevertheless a free and a basal surface, the latter in organic combination with the base. Thus, here again we are presented with a case of distinct polar differentiation of the skin epithelium, which is manifested by the presence of three layers.

But furthermore, the process of shedding, which under normal conditions represents the result of vertical growth and goes hand in hand with the replacement of cells originating from the basal layers, is reintroduced, thus completing the resemblance between explanted and normal epithelium. It is true that so far we have not succeeded in discovering any examples of mitosis in the basal cells; but inasmuch as only very few of the epithelial spheres have so far been examined in sections, we must refrain from stating positively by what means the giving off of very considerable numbers of cells is rendered possible.

SUMMARY.

1. Fragments of skin taken from the back of the leopard frog were cultivated in a mixture of plasma and muscle extract of the same species.

2. A few hours after explantation, processes of activity are seen to arise, which finally lead to the formation of a compact epithelial rim around the fragment of skin.

3. These epithelial cells undergo gradual transformation into a spindle-shaped type of cell; in this form they resemble the spindle cells which have been described as connective tissue cells.

4. The growth of a rim of tissue around the explanted fragments of skin may be ascribed principally to the activity of a basal layer of epithelial cells, the units of which first advance into the medium as compact membranes by means of the so called epithelial movement, but which later become separated from the compact membrane, and having assumed a spindle form, spread according to the manner of connective tissue cells.

5. In contradistinction to this basal layer, the cells of an upper and middle layer of epithelium upon separation from the fragment of skin and isolation in the nutritive medium, remain completely inactive.

6. The connective tissue of frog skin, for reasons not yet completely established, does not participate in the production of the rim of cells, possibly for the reason that it was early surrounded by the epithelium and was thus prevented from sending out cells into the medium.

7. The transformation of the epithelial cells into a type of cells of the spindle form is a result of the changes in the physicochemical conditions brought about by the life *in vitro*, which become similar to the physicochemical conditions normally characteristic of connective tissue.

8. The final stage of the explanted fragments of skin is a hollow epithelial sphere (cystic stage) filled with connective tissue.

9. During the cystic stage the epithelium again shows its normal polar differentiation, as a consequence of the physicochemical conditions which now approach normal.

10. These conditions also permit of the shedding of cells in a vertical direction, although previously the production of cells in a horizontal direction had already become impossible.

EXPLANATION OF PLATES.

Plate 32.

Fig. 1. Primary epithelial rim. A rim of pure epithelial tissue surrounding the original fragment of skin. The small dots are cell nuclei, the large black specks in the epithelial rim (especially clear on the lower part of the figure) are the small melanophores of the epithelium. Photograph 20, culture A 429; stained with hematoxylin; magnification, 60; three hours after explantation.

Fig. 2. Secondary epithelial rim. The epithelial character of the tissue (polygonal form of the cells) can be clearly discerned, as well as the large cell nuclei. The small, black specks which are sometimes extended into long processes are the small melanophores. On three sides of the original fragment the central zone of the secondary epithelial rim can be seen. It is a little darker than the peripheral zone. Photograph 22, culture B 13; photographed while alive; forty-eight hours after explantation into a medium composed of frog plasma, chicken plasma, and frog muscle extract.

PLATE 33.

FIG. 3. Secondary epithelial rim, after the uppermost and middle layers of epithelium have been discarded. As the middle layer with the small melanophores was absent during the growth of the secondary epithelial rim, the small melanophores are absent in the latter. Moreover, the figure also shows the beginning of the loosening up process; jagged processes consisting of long drawn out cells are entering the medium. Photograph 2, culture A 430 a; photographed while alive; magnification, 80; six days after explantation.

FIG. 4. Loosening up. This shows a greatly magnified section of the rim of the culture illustrated in figure 3. In the epithelial skin consisting of polygonal cells a gap can be seen spanned by a long drawn out cell. Photograph 3, culture A 430 a; photographed while alive; magnification, 350; six days after explantation.

PLATE 34.

FIG. 5. Secondary activity. The tertiary epithelial rim, formed after the dissolution of the secondary epithelial rim, is here likewise in process of dissolution. But on the left and right the epithelial structure can still be clearly made out. On the other hand, rings of cells have formed above and below, which are partially connected with the original fragment by means of columns of cells. One of them (above) has just become torn; the cells at the center have become spherical in form. Photograph 1, culture A 406; photographed while alive; six days after explantation.

PLATE 35.

FIG. 6. Loosening up in very fluid plasma. A wide ring of cells surrounds the fragment, with which it is connected by means of columns of cells. Culture A 405; drawn while alive; magnification, Leitz ocular 1 + objective 3; thirty hours after explantation.

PLATE 36.

FIG. 7. Columns of cells of a culture undergoing loosening up. Culture A 126; drawn while alive; magnification, Leitz ocular 1 + objective 6; twenty-four hours after explantation.

PLATE 37.

FIG. 8. Loosening up. The same culture as in figure 3, one day later. In several places the transformation of the epithelial skin into protuberances consisting of long spindle-shaped cells can clearly be seen. Photograph 7, culture A 430 a; photographed while alive; magnification, 60; seven days after explantation.

PLATE 38.

FIG. 9. Spindle-shaped epithelial cells. Taken from a culture in the process of loosening up. Photograph 19, culture A 214; stained with hematoxylin; magnification, 350; forty-eight hours after explantation.

PLATE 39.

FIG. 10. Figure 11, more highly magnified. The large polygonal cells of the uppermost layer can be seen through the middle layer. Photograph 5 a, culture A 431 b; photographed while alive; magnification, 370; three days after isolation.

Plate 40.

Fig. 11. Uppermost and middle layers of epithelium, separated from the explanted fragment of skin and viewed from beneath. The small, polygonal cells of the middle layer can be seen, as well as the greatly contracted small melanophores, which appear as small specks. On the upper edge some cells have advanced into the medium, but all the other cells have remained inactive. Photograph 4, culture A 431 b; photographed while alive; magnification, 120; three days after isolation.

Fig. 12. Epithelial cyst. At the lowest rim of the sphere originating from the explanted fragment of skin a layer consisting of cells in the act of becoming detached can be discerned. Photograph 10, culture A 406 d; photographed while alive; slight magnification; eighteen days after explantation.

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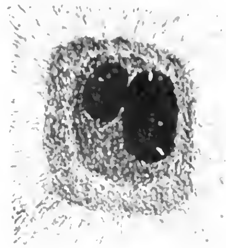


FIG. 1.

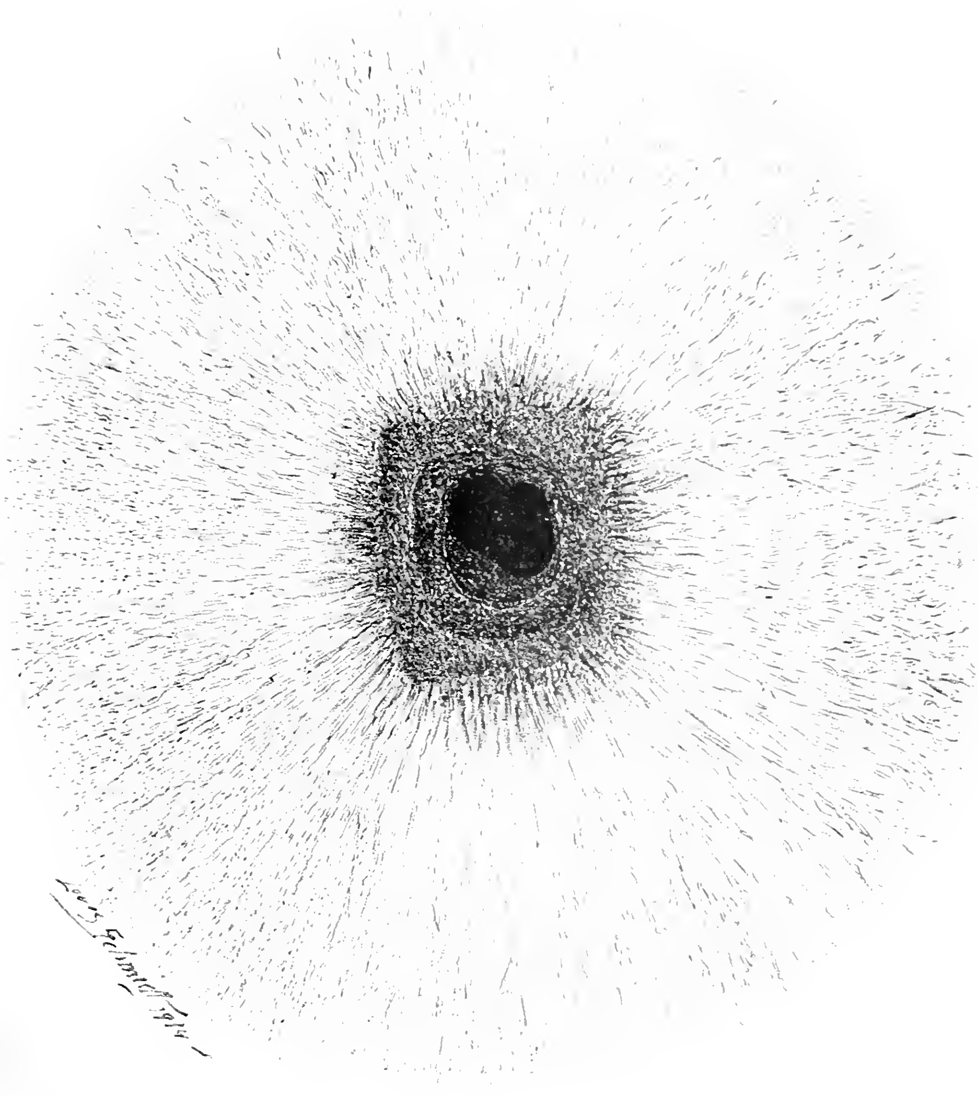


FIG. 2.

(Carrel: Condition of Strain of Connective Tissue.)

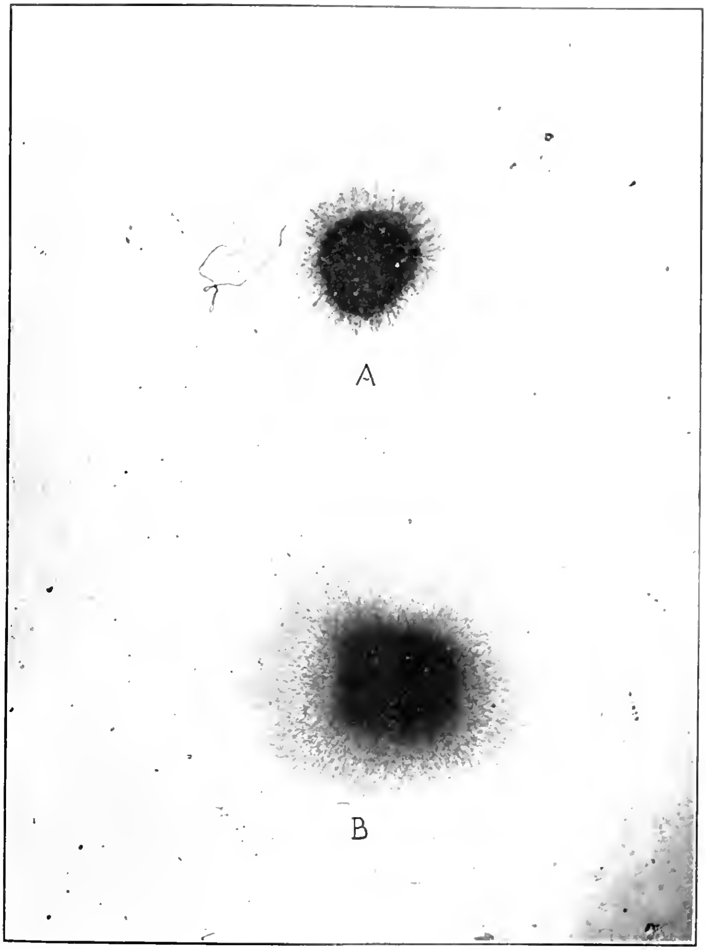


Fig. 3

[illegible] Condition of Strain of Connective Tissue

FIG. 1.

(Carrel: Operations on Sigmoid Valves of Pulmonary Artery.)

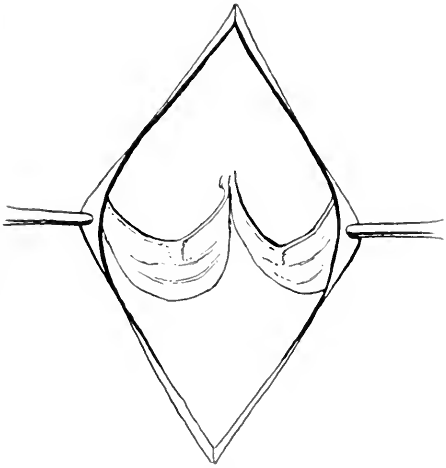


FIG. 2.

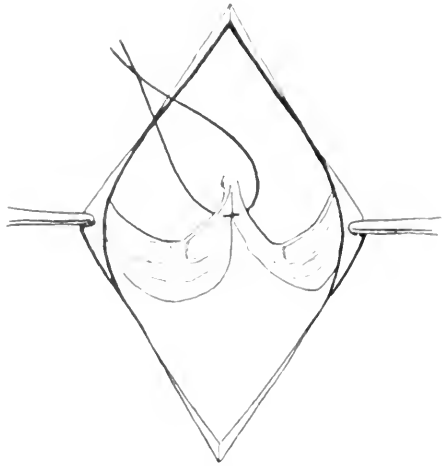


FIG. 3.

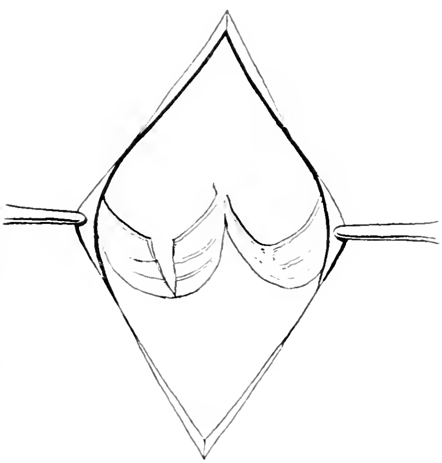


FIG. 4.

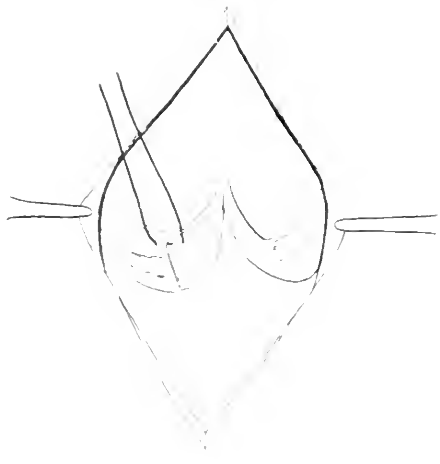


FIG. 5.

(Carrel: Operations on Sigmoid Valves of Pulmonary Artery.)

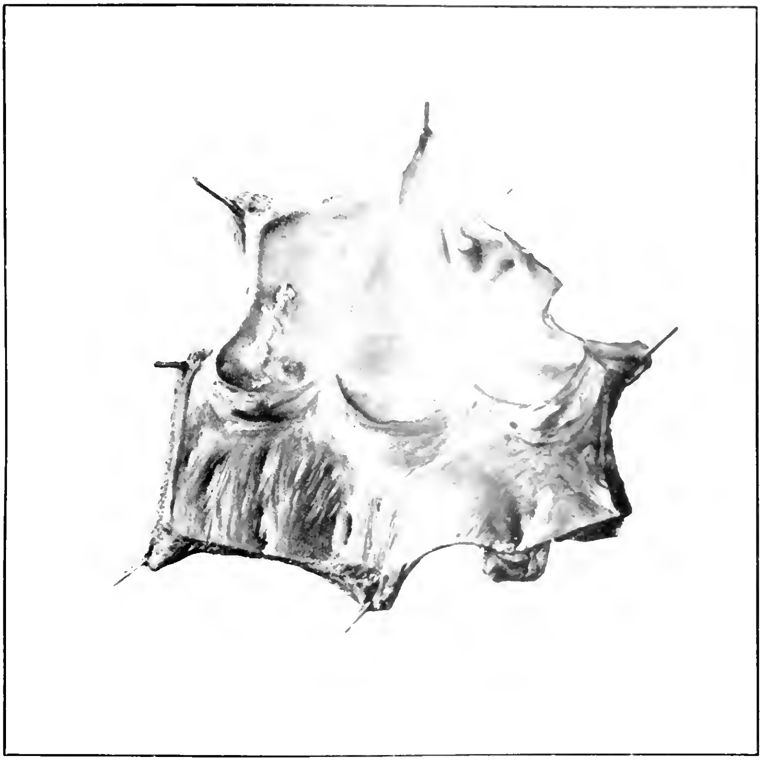


FIG. 6

(Carrel: Operations on Sigmoid Valves of Pulmonary Artery.)

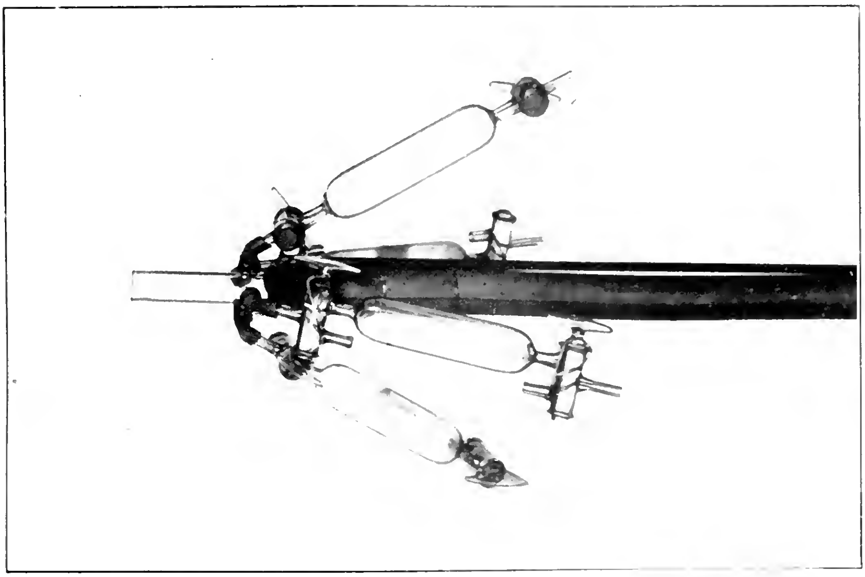


Fig. 1.

(Carter: Calculation of Dead Space in Breathing.)

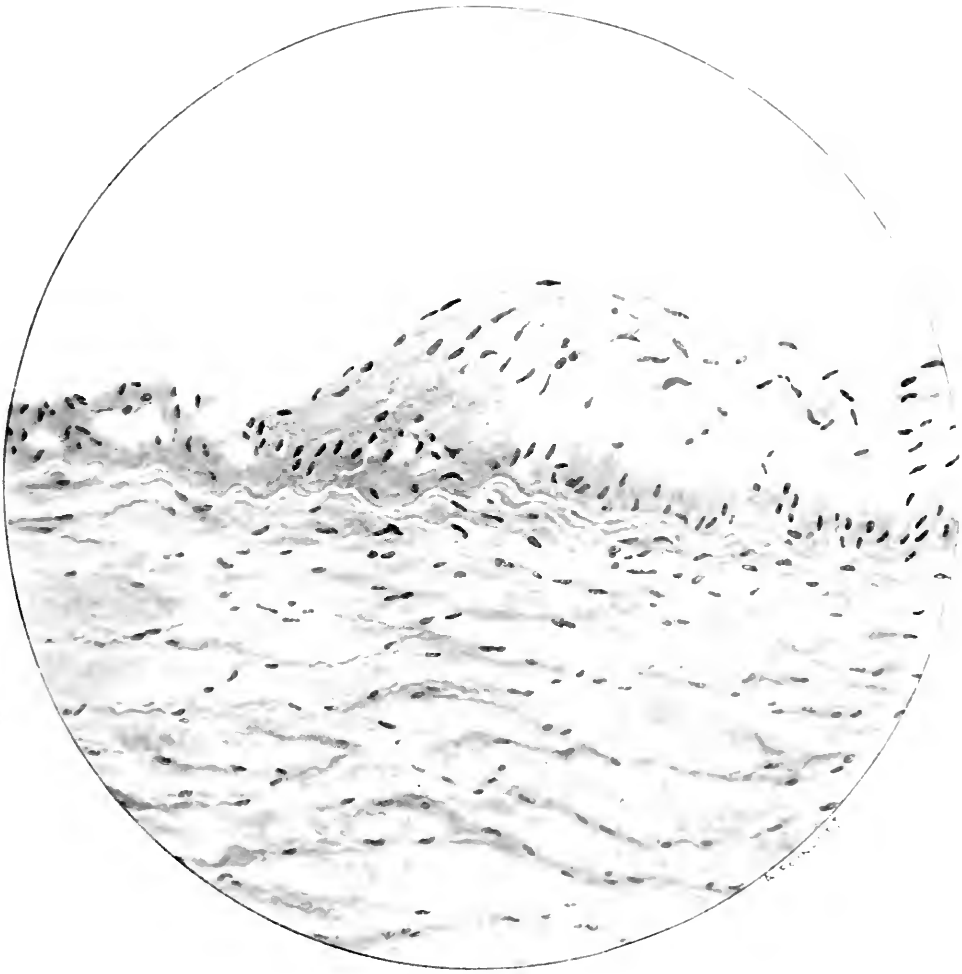


FIG. 1.

Adler: Studies in Experimental Atherosclerosis.

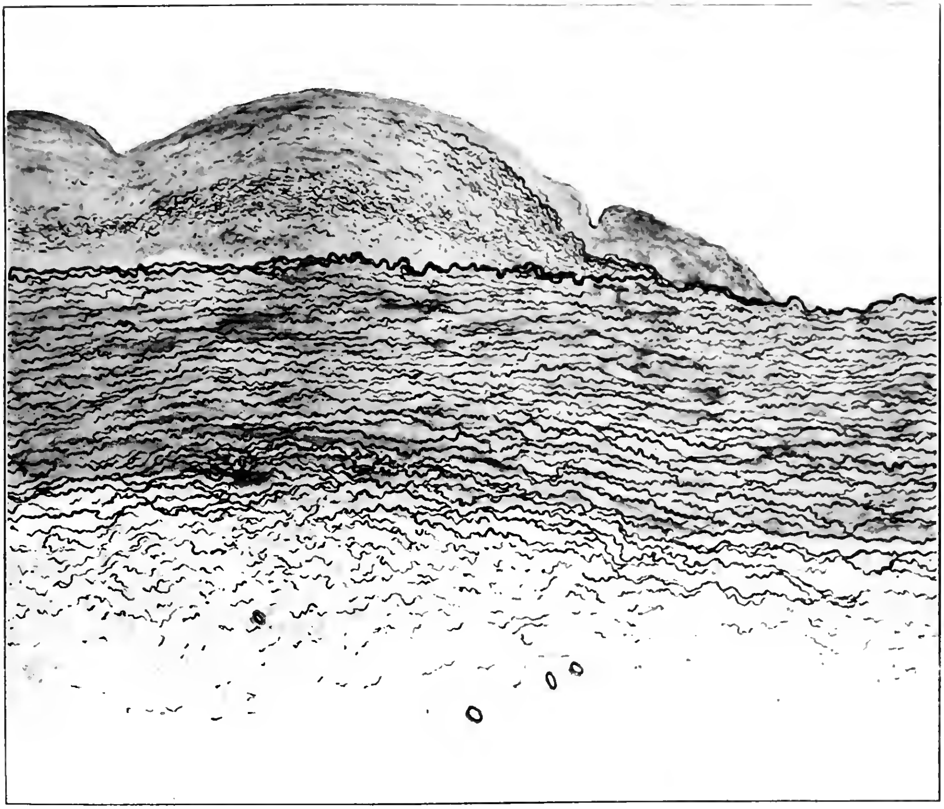


FIG. 2.

(Adler: Studies in Experimental Atherosclerosis.)

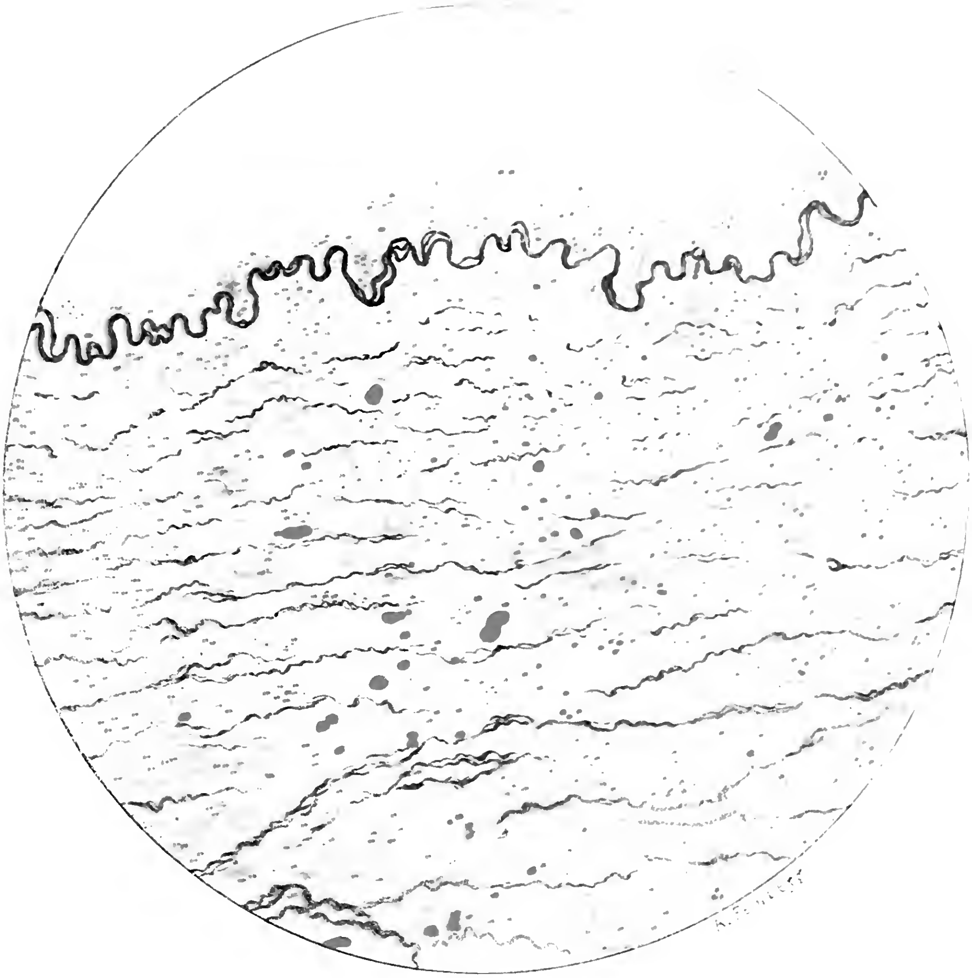


Fig. 3.

Adler: Studies in Experimental Atherosclerosis.

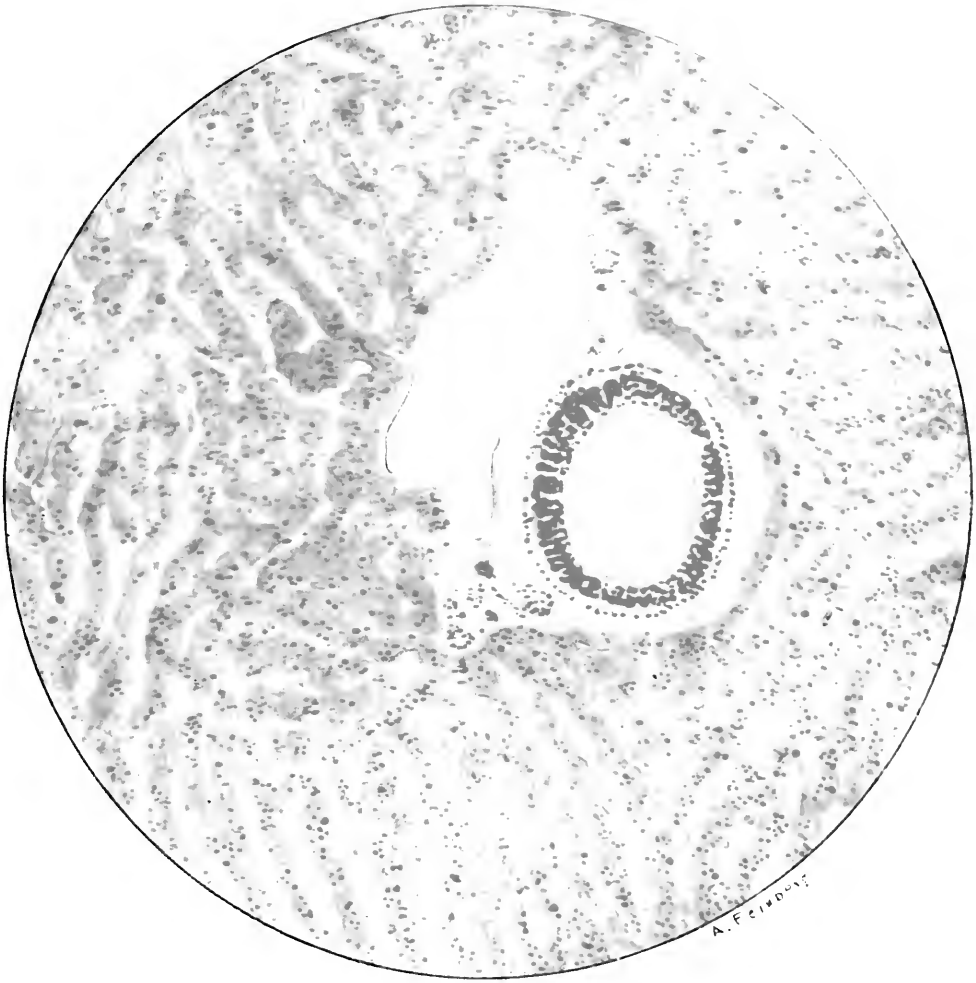


FIG. 4.

Adler: Studies in Experimental Atherosclerosis.

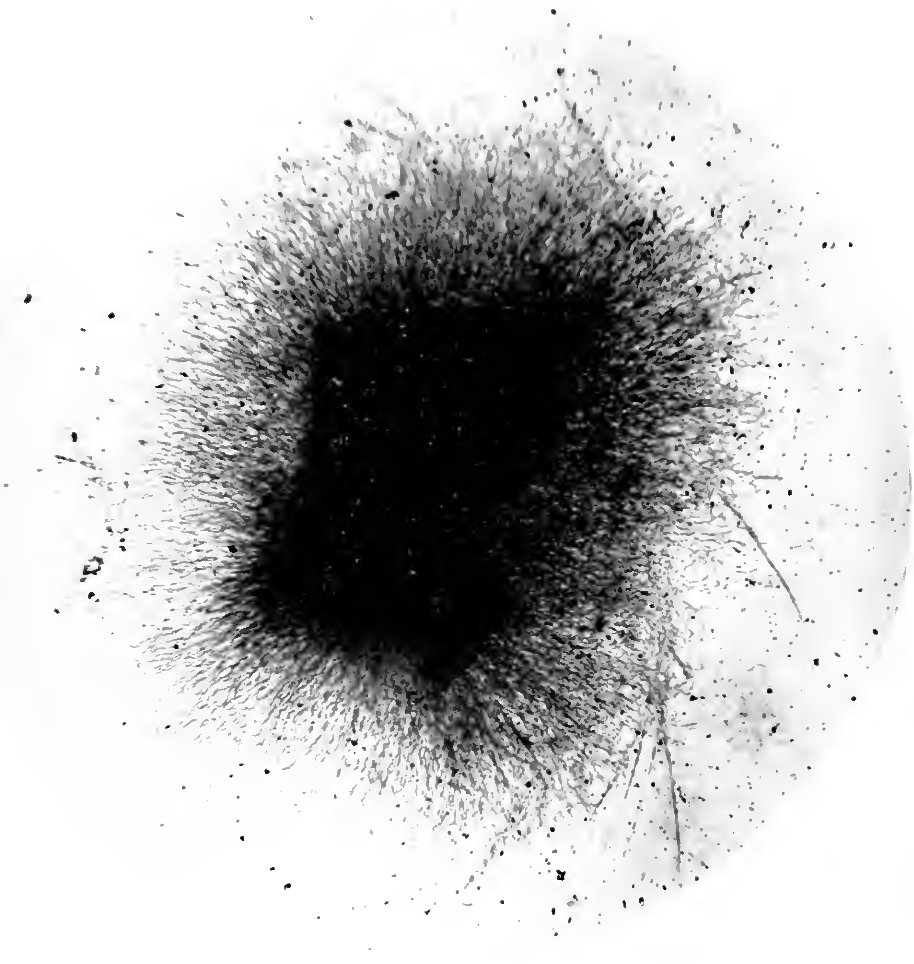


FIG. 1.

(Ebeling: Cell Proliferation of Connective Tissue.

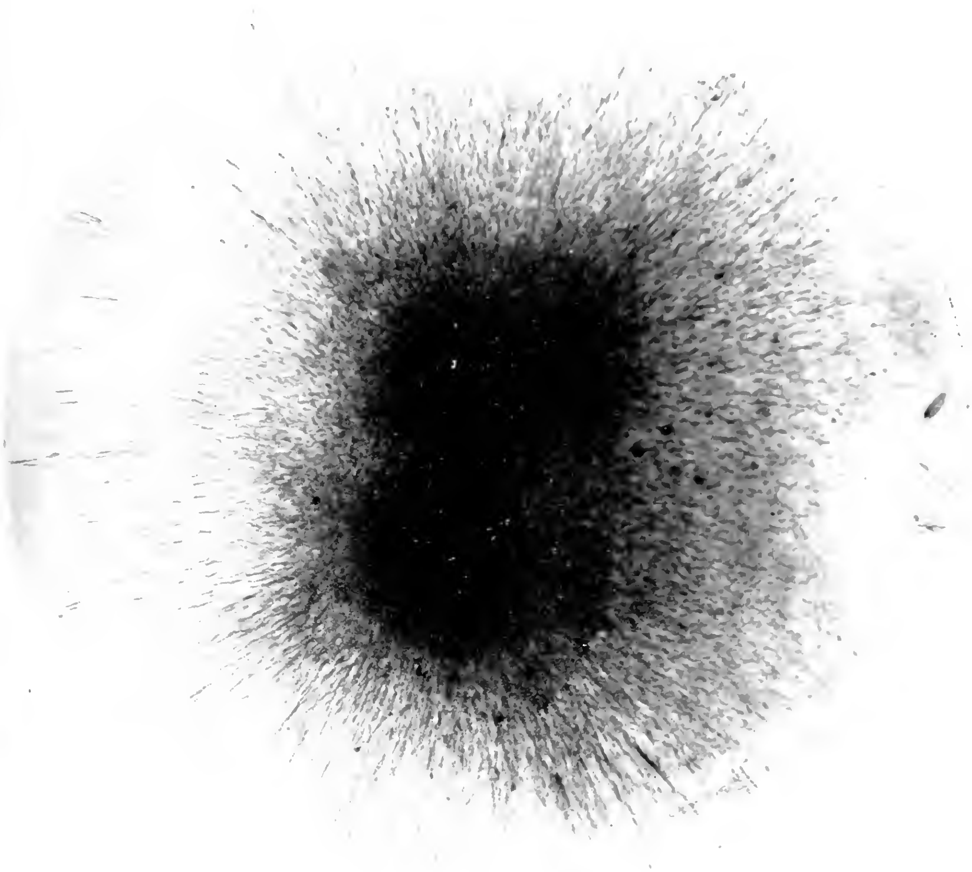


FIG. 2.

(Ebeling: Cell Proliferation of Connective Tissue.)

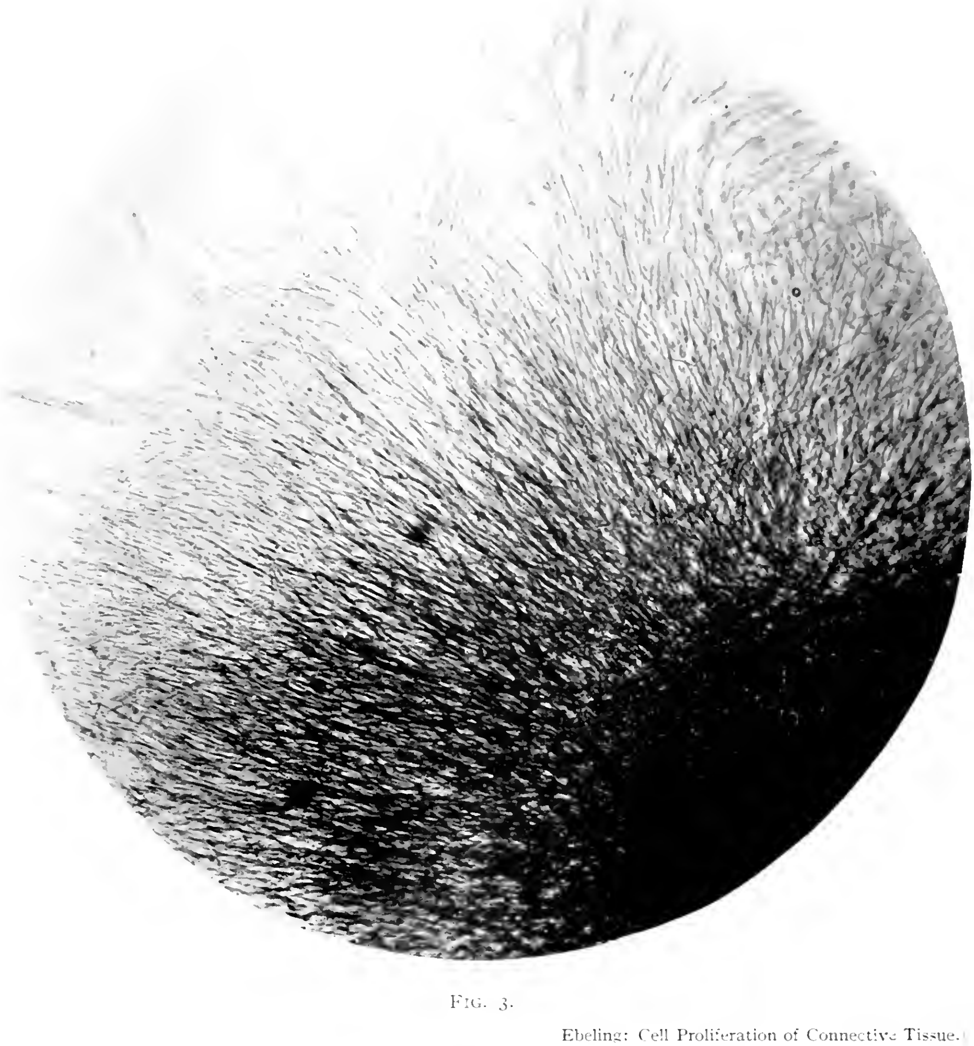


FIG. 3.

Ebeling: Cell Proliferation of Connective Tissue.)

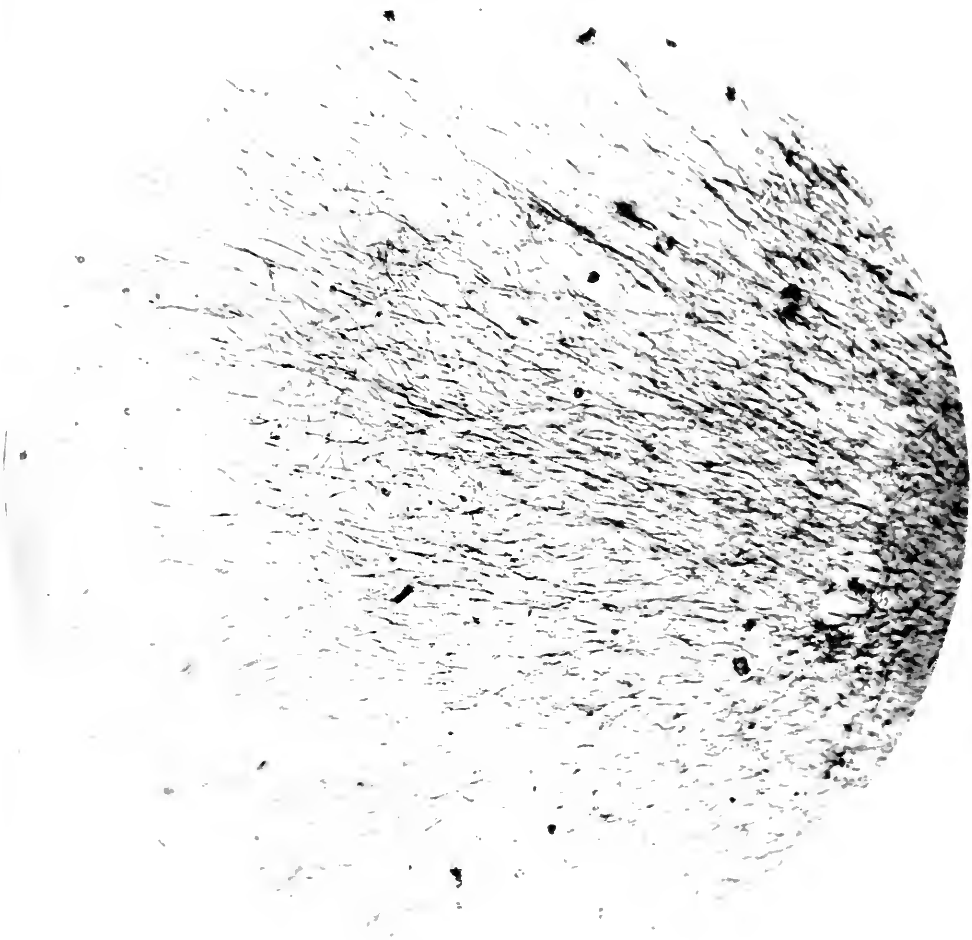


FIG. 4.

(Ebeling: Cell Proliferation of Connective Tissue.)

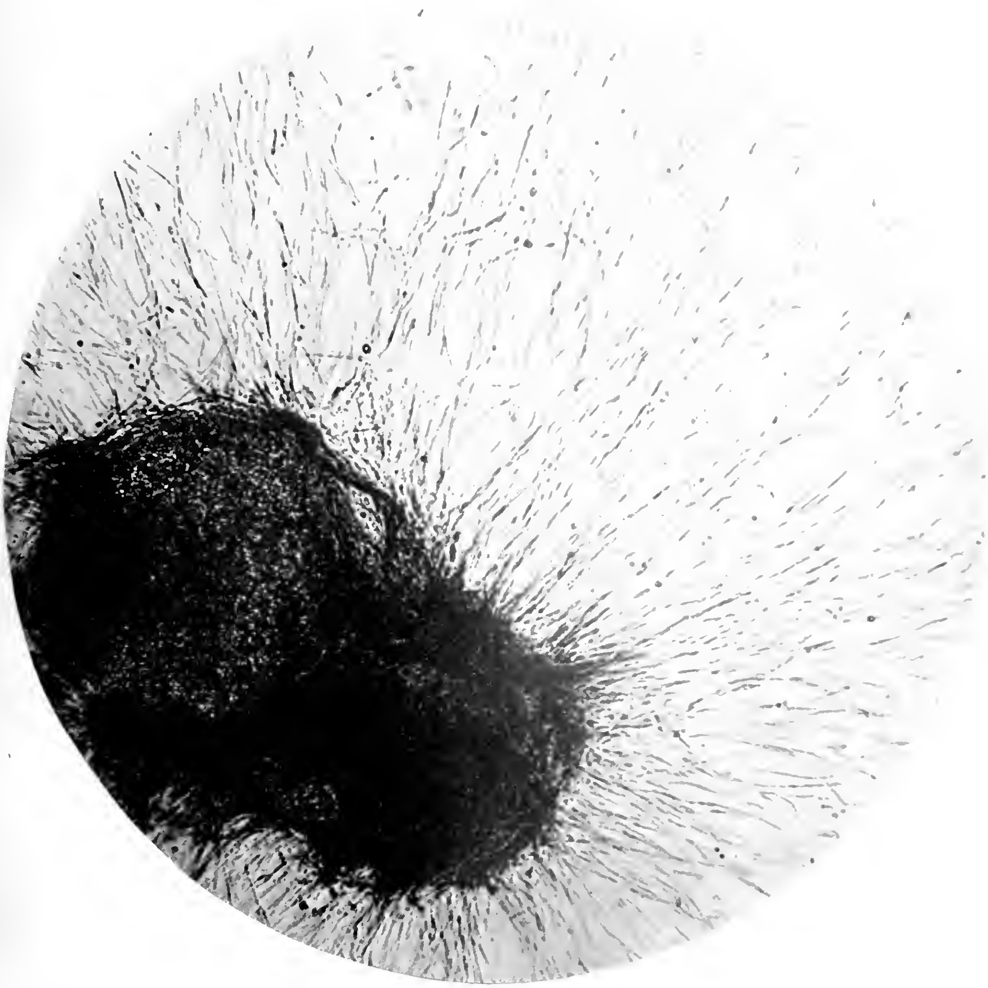


Fig. 1.

(Losee and Ebeling: Cultivation of Human Sarcomatous Tissue *in Vitro.*)

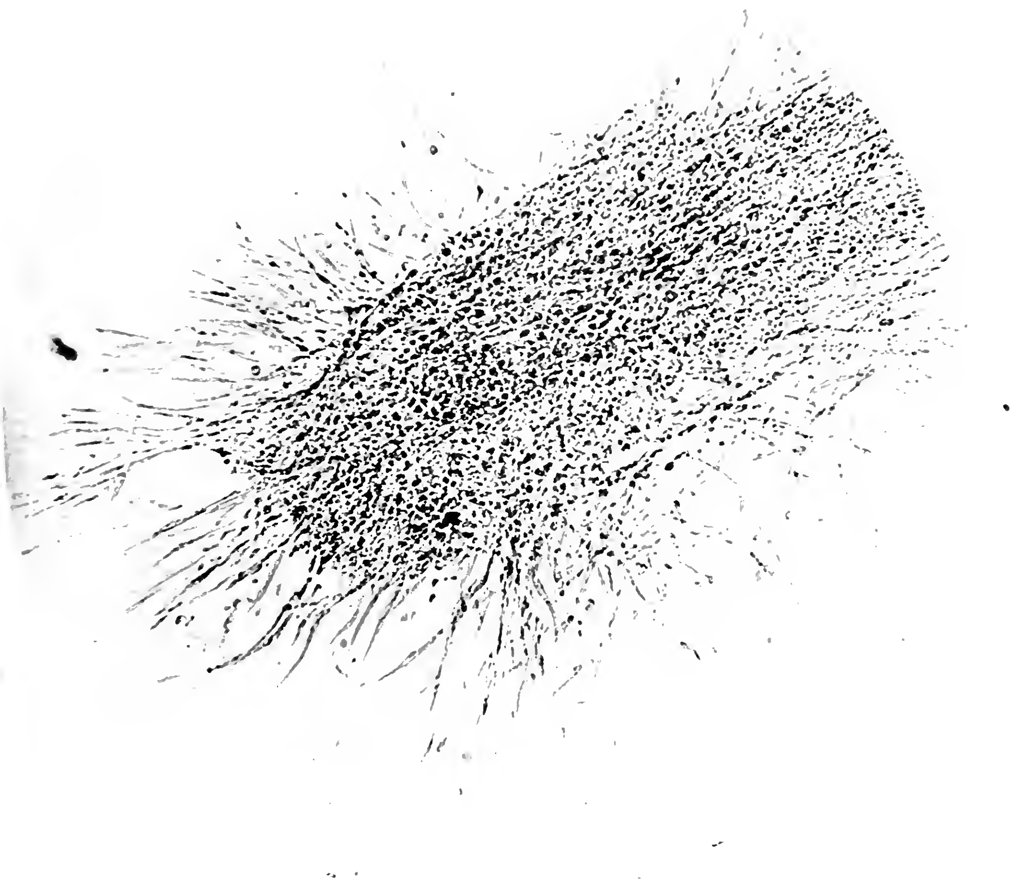


FIG. 2.

(Losee and Ebeling: Cultivation of Human Sarcomatous Tissue *in Vitro*.)

FIG. 3.

(Losee and Ebeling: Cultivation of Human Sarcomatous Tissue *in Vitro*.)

FIG. 1.

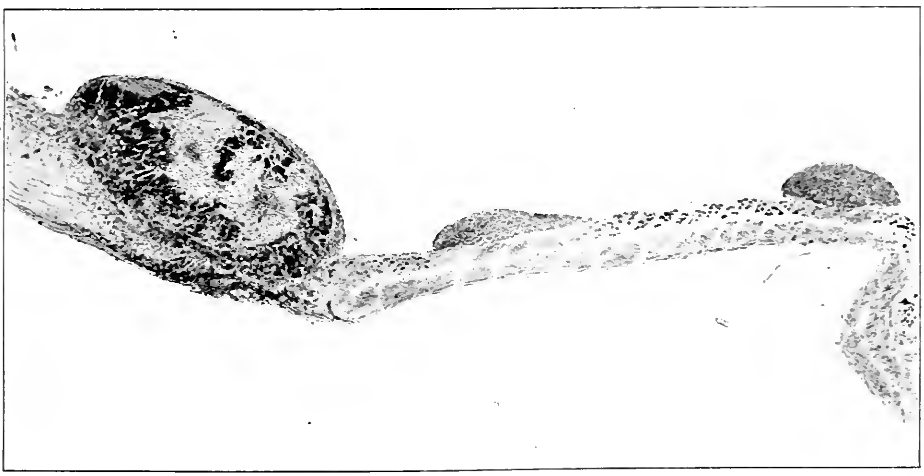


FIG. 2.

(Jones and Rous: Localization of Secondary Tumors.)

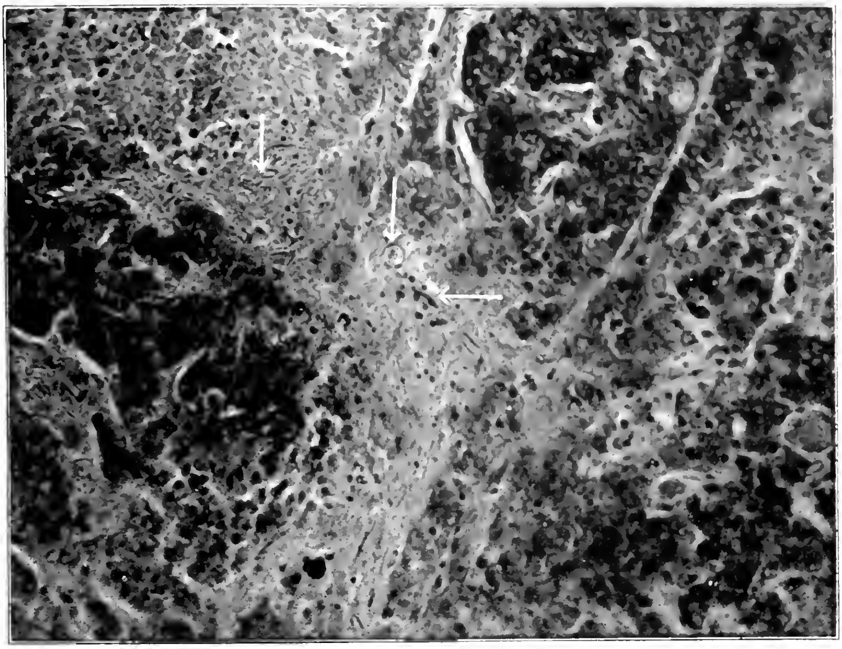


FIG. 3.

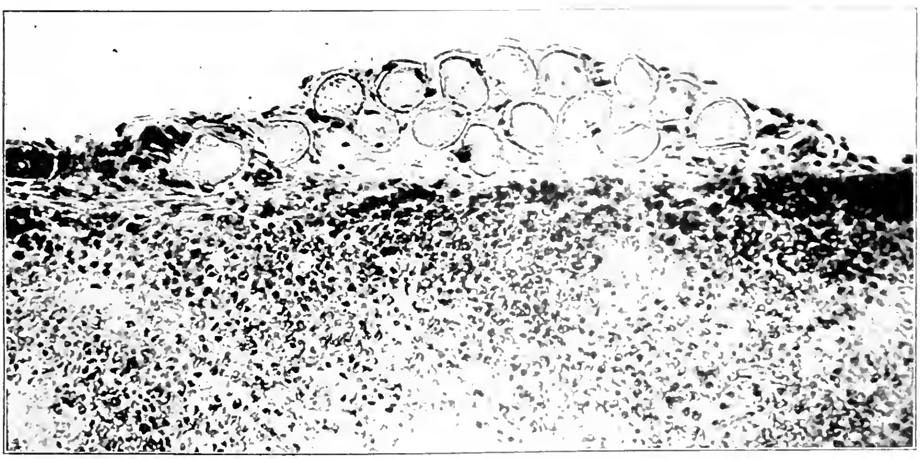


FIG. 4.

(Jones and Rous: Localization of Secondary Tumors.)

FIG. 5.

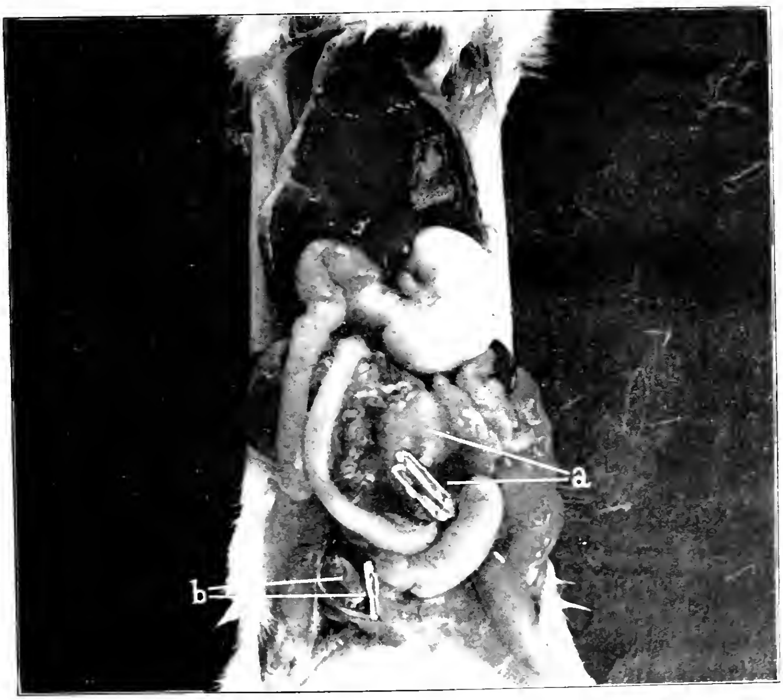


FIG. 6.

(Jones and Rous: Localization of Secondary Tumors.)

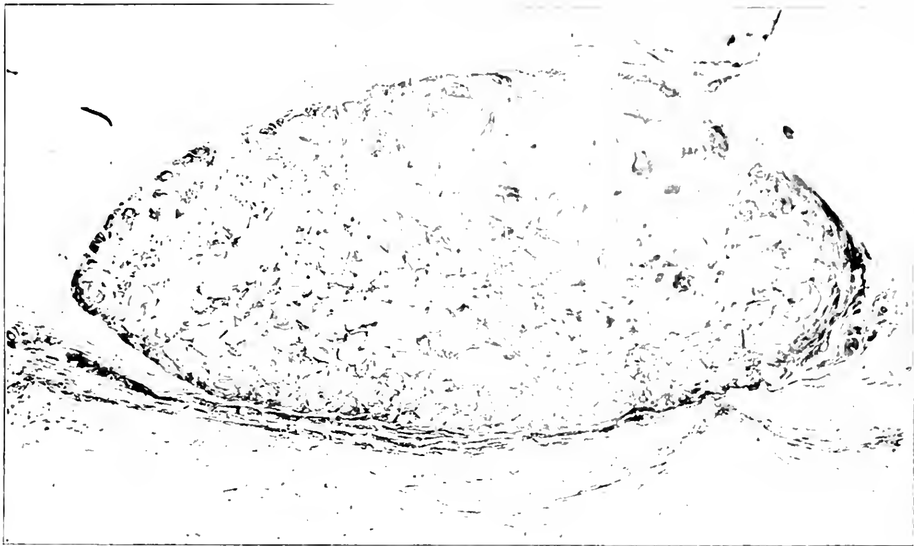
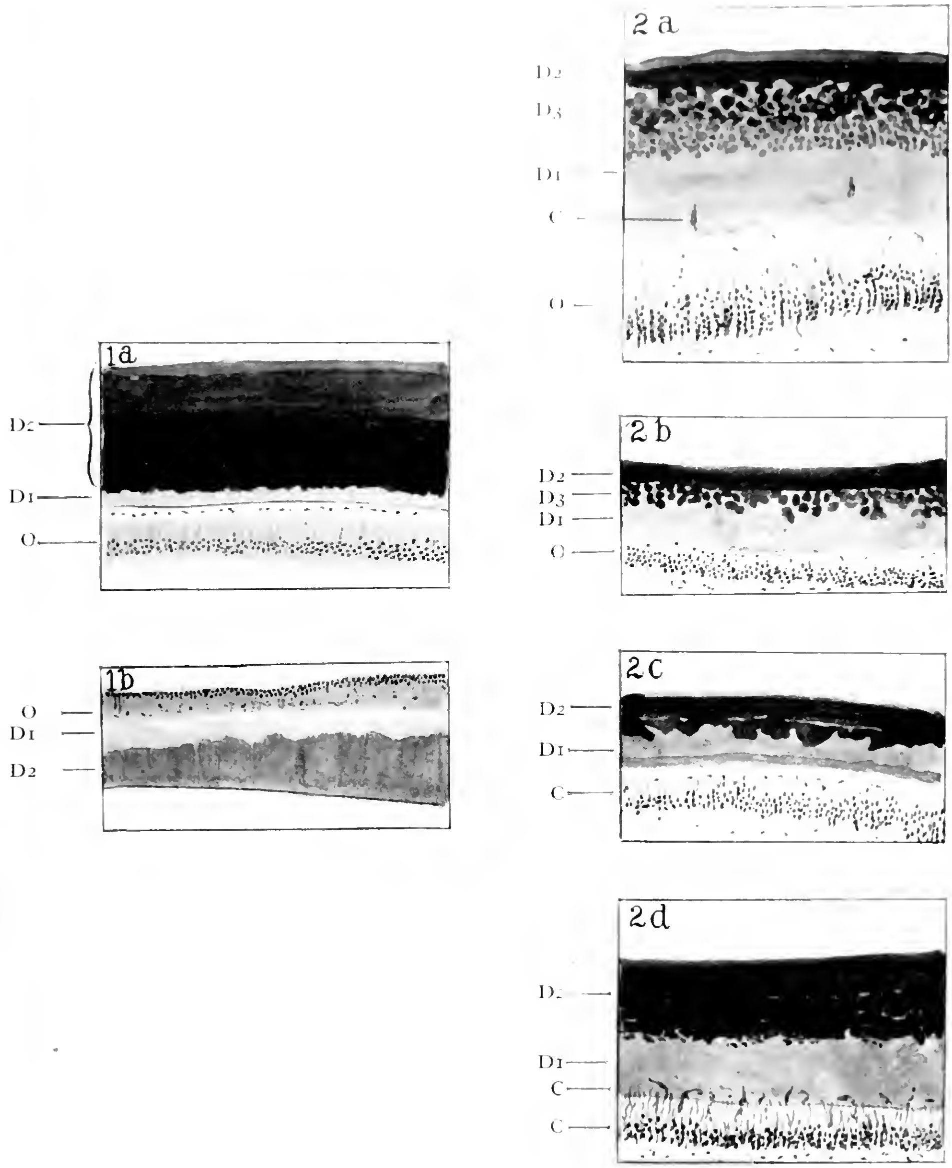


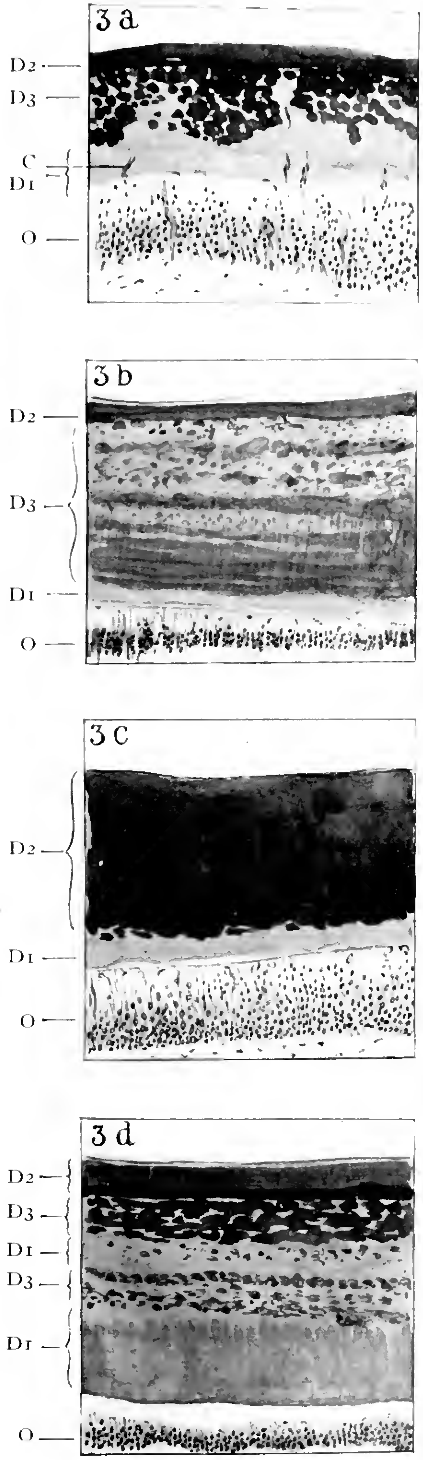
FIG. 1.

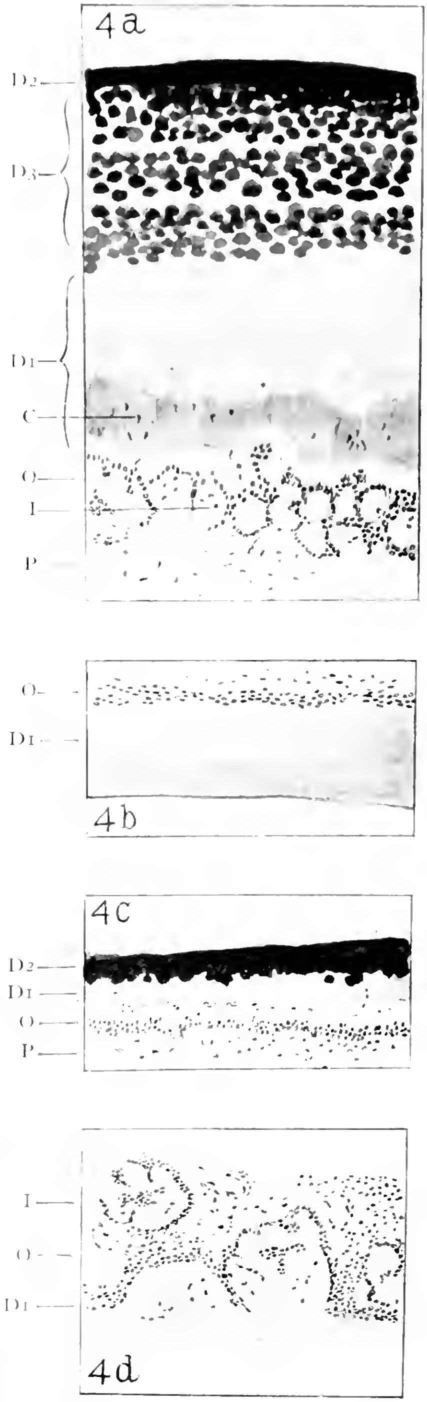
FIG. 2.

(Rous: Influence of Diet on Tumors.



(Pappenheimer: Effects of Extirpation of Thymus in Rats.)





(Pappenheimer: Effects of Extirpation of Thymus in Rats.)

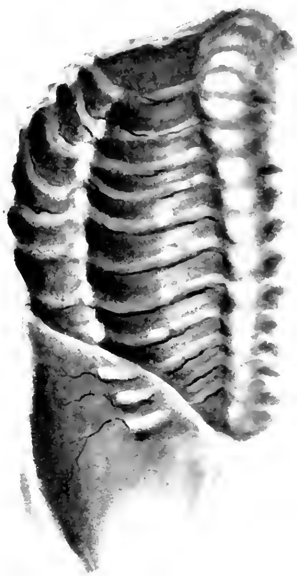


Fig. 5.

(Pappenheimer: Effects of Extirpation of Thymus in Rats.)

FIG. 6.

(Pappenheimer: Effects of Extirpation of Parathyroids in Rats.)

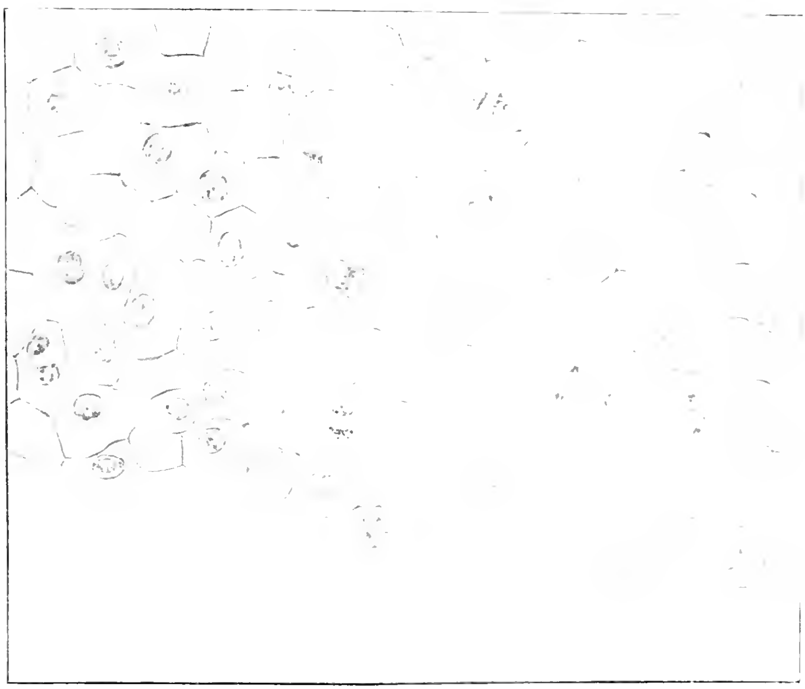
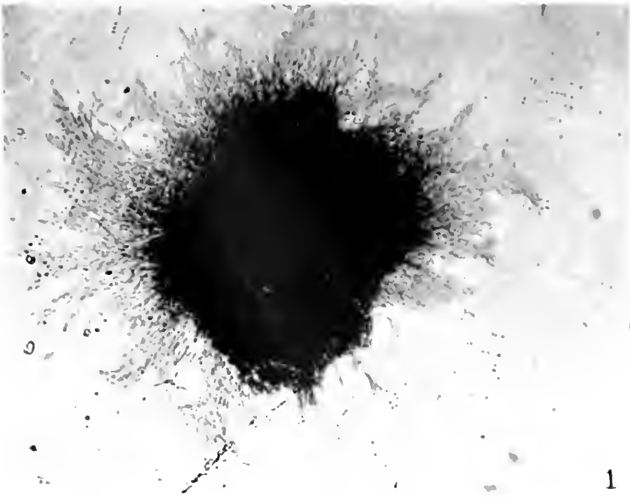


FIG. 1.

(Russell: Effect of Gentian Violet on Protozoa.)



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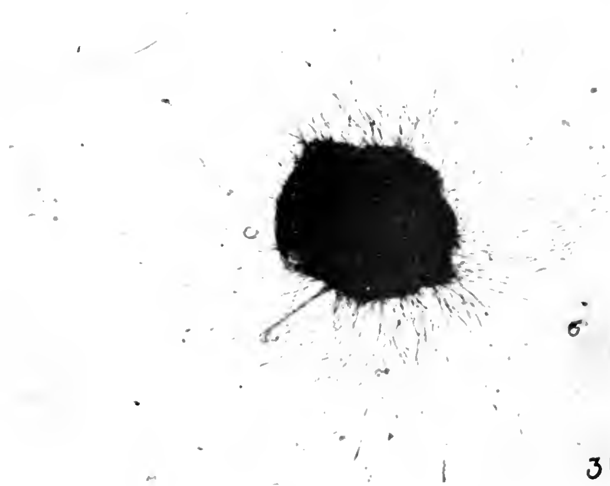
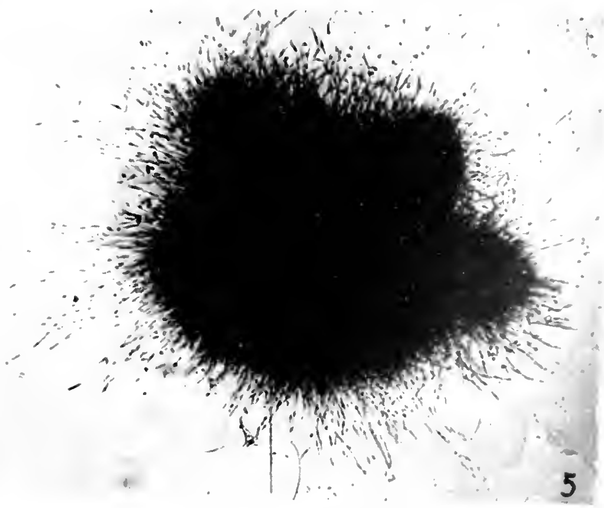


3

(Walton: Growth of Adult Mammalian Cells *in Vitro*.)

4



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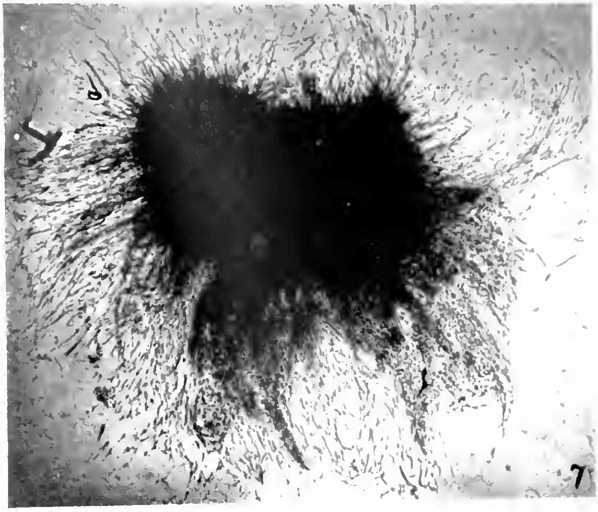
6

(Walton: Growth of Adult Mammalian Cells *in Vitro*.)



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(Walton: Growth of Adult Mammalian Cells *in Vitro*.)

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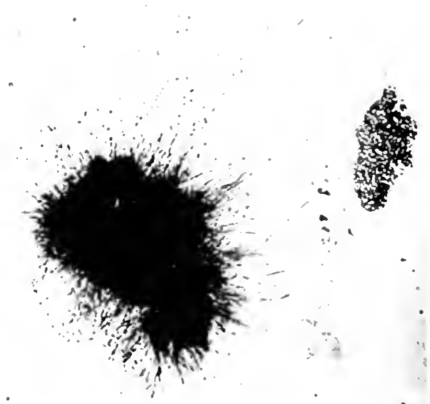
12

(Walton: Growth of Adult Mammalian Cells *in Vitro*.)

13



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15

(Walton: Growth of Adult Mammalian Cells *in Vitro*.)

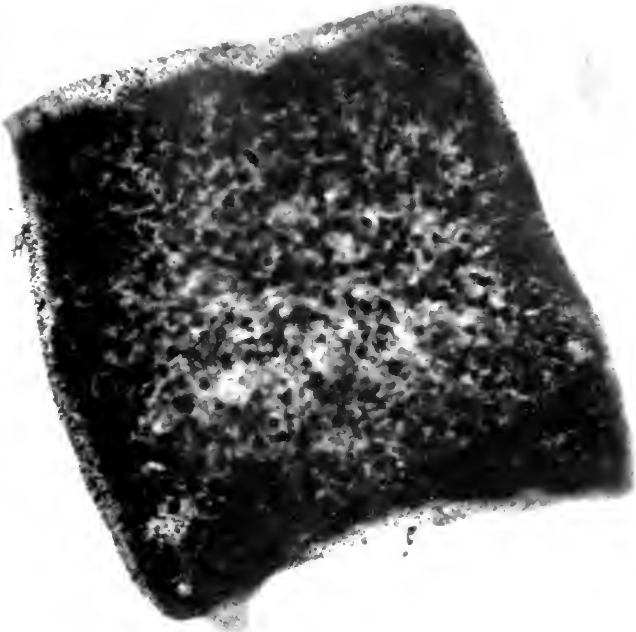

FIG. 1.

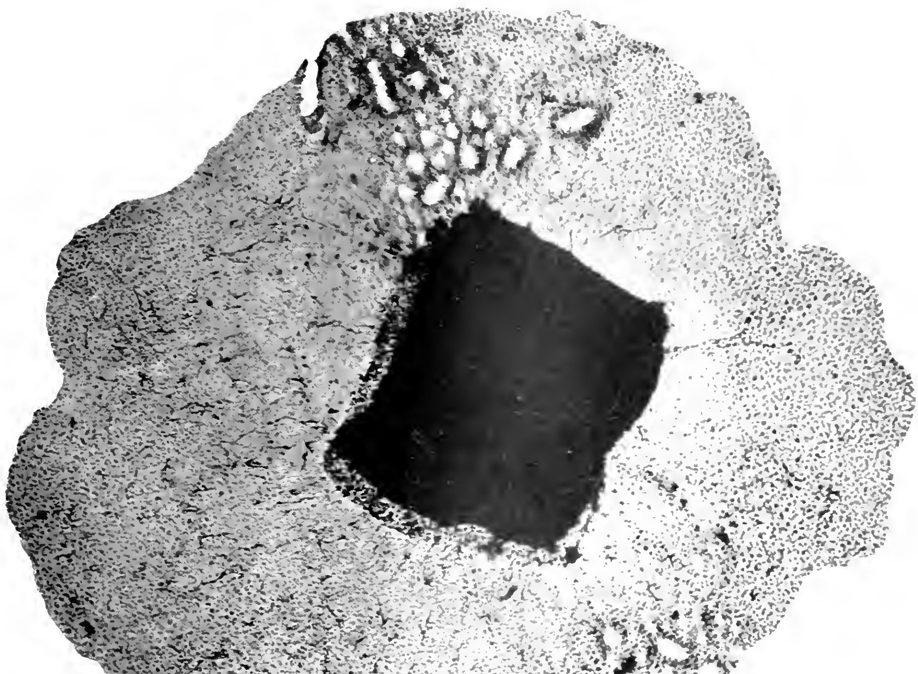

FIG. 2.

(Uhlenhuth: Cultivation of Skin Epithelium of Frog.)

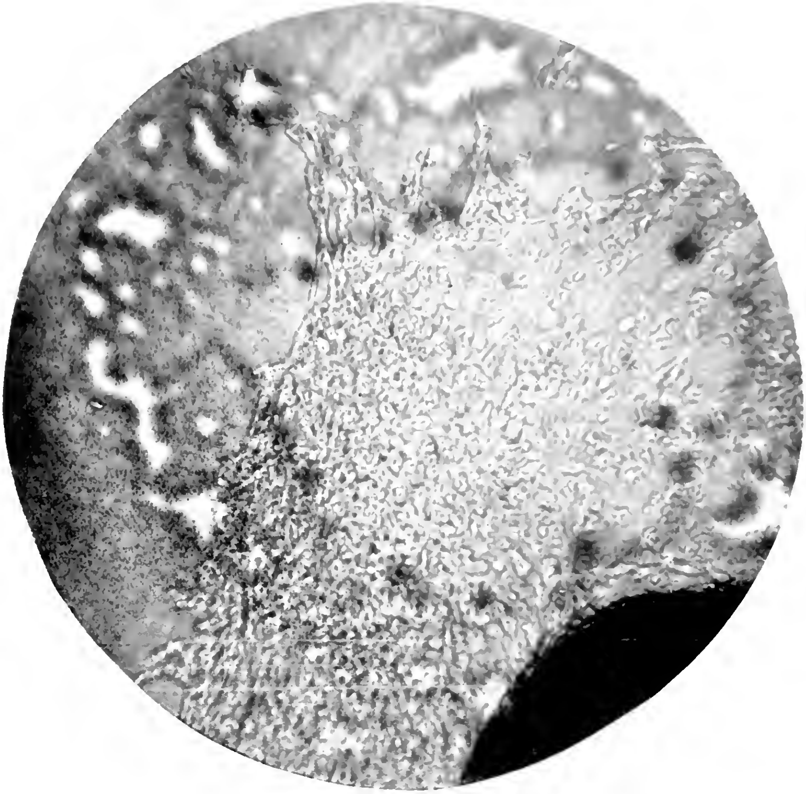

FIG. 3.

FIG. 4.

(Uhlenhuth: Cultivation of Skin Epithelium of Frog.)

FIG. 5.

(Uhlenhuth: Cultivation of Skin Epithelium of Frog.)

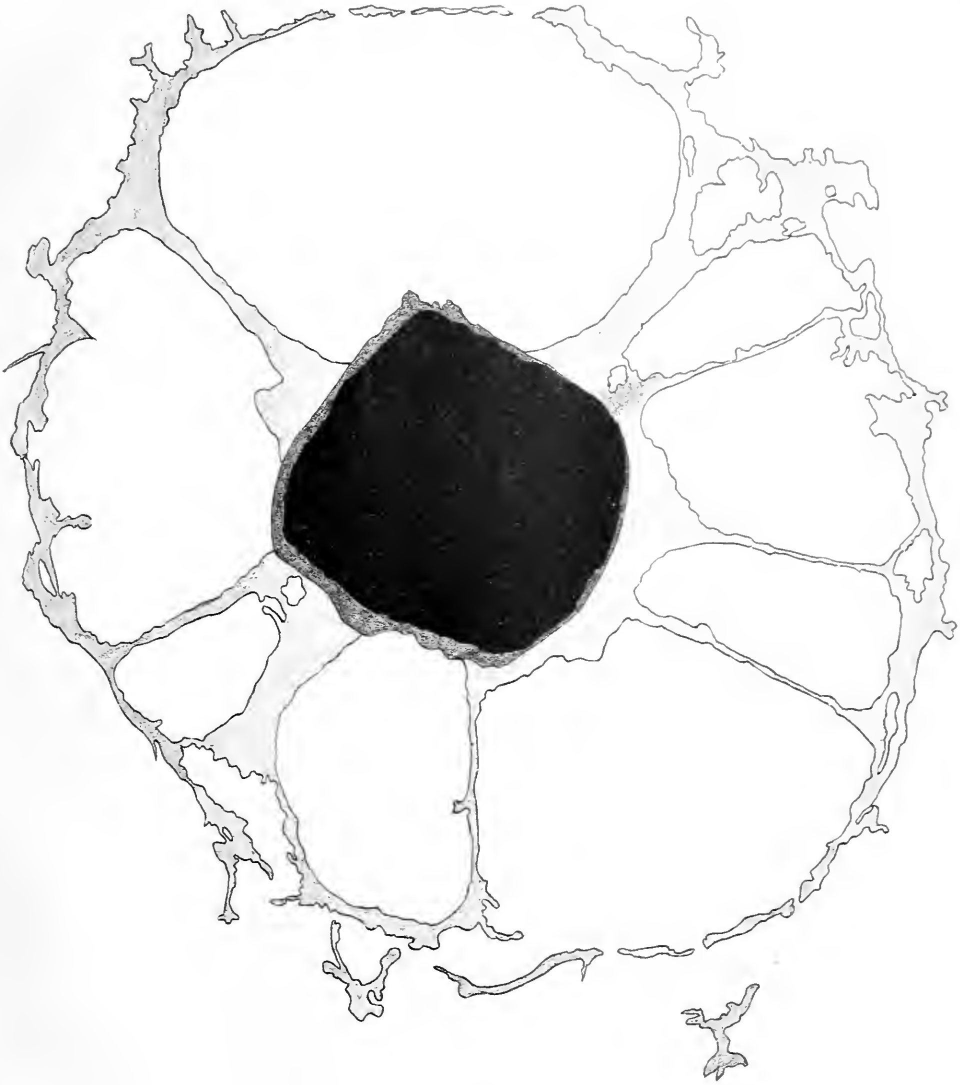

FIG. 6.

(Uhlenhuth: Cultivation of Skin Epithelium of Frog.)

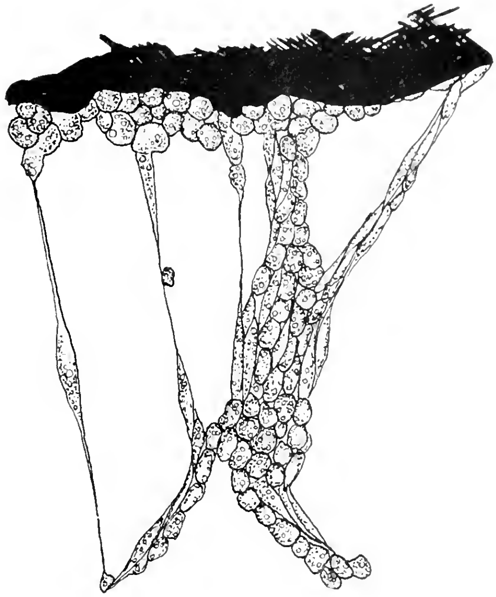

FIG. 7.

(Uhlenhuth: Cultivation of Skin Epithelium of Frog.)

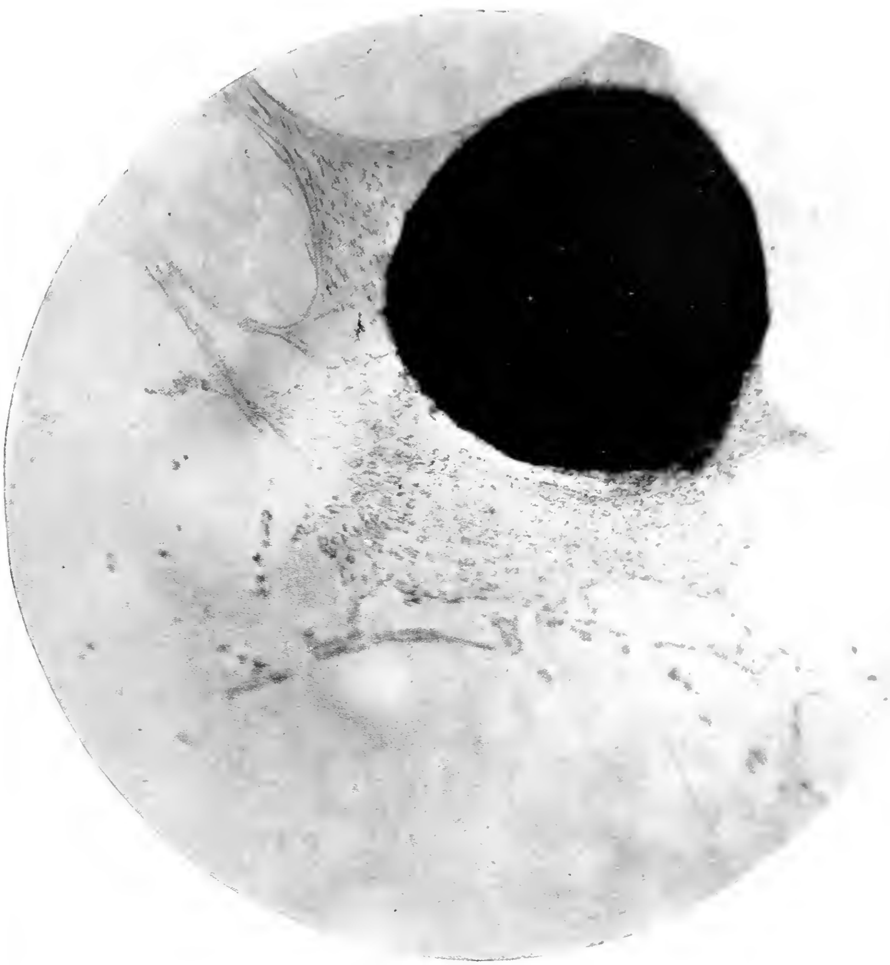

FIG. 8.

(Uhlenhuth: Cultivation of Skin Epithelium of Frog.)

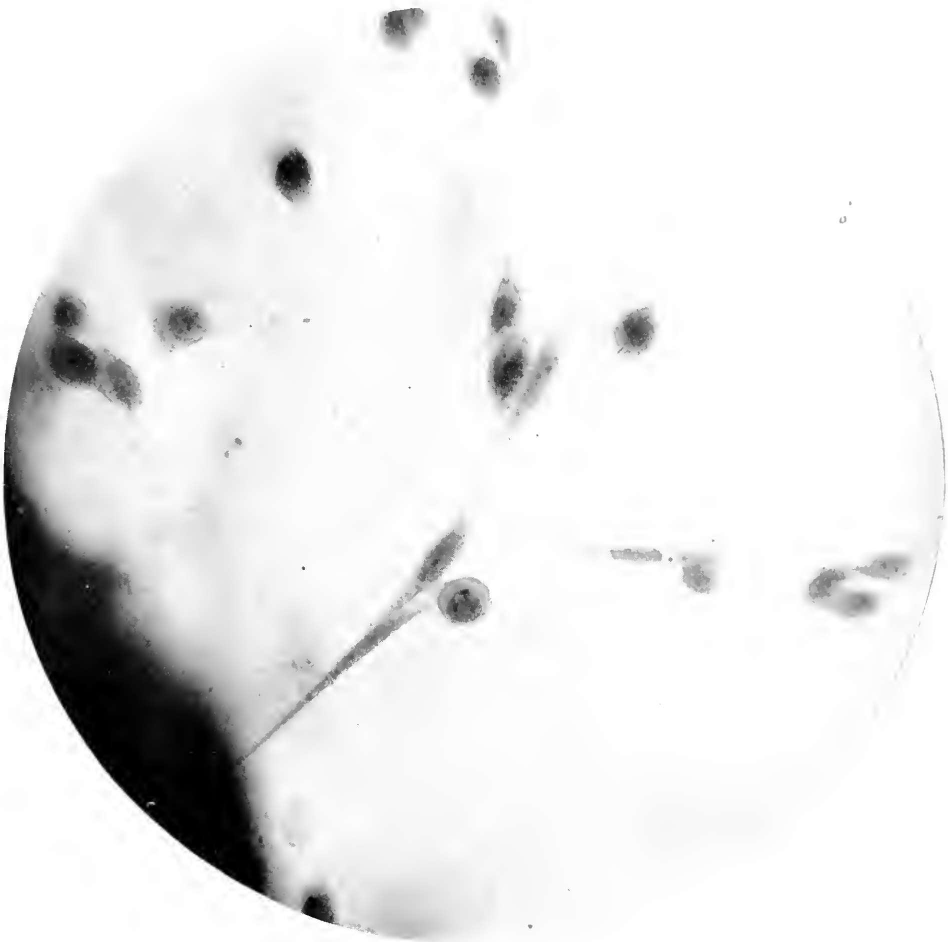

Fig. 9.

(Uhlenhuth: Cultivation of Skin Epithelium of Frog.)

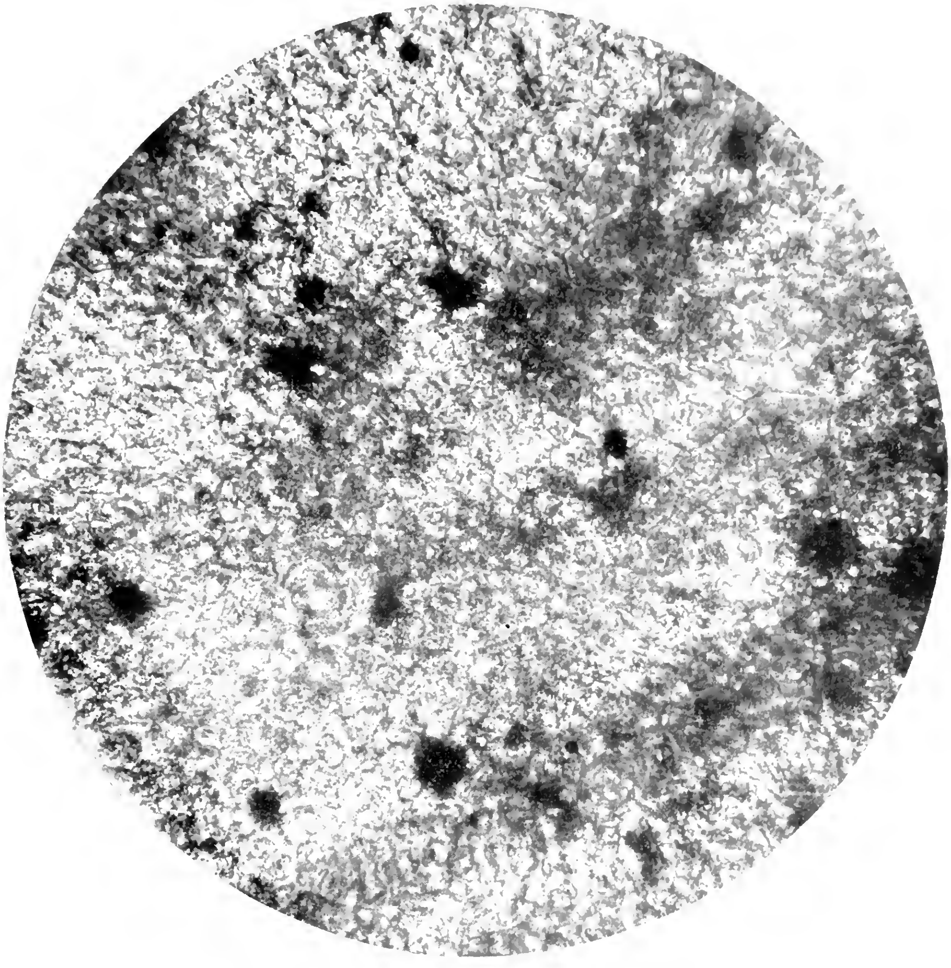

FIG. 10.

(Uhlenhuth: Cultivation of Skin Epithelium of Frog.)

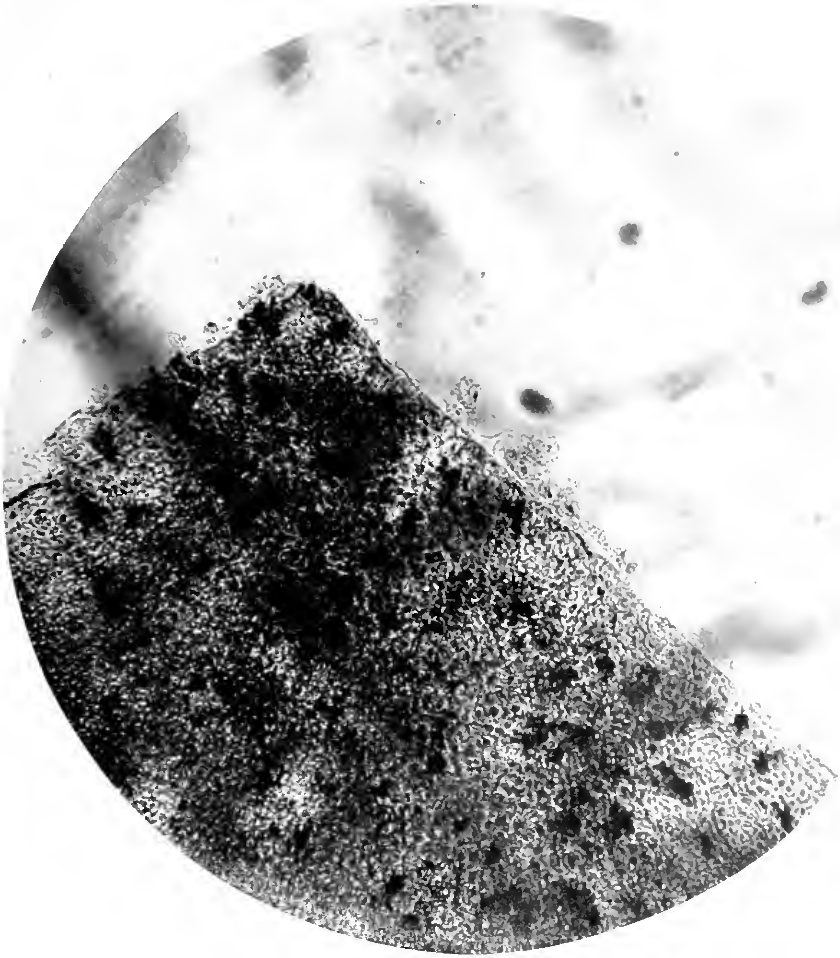

FIG. 11.

FIG. 12.

(Uhlenhuth: Cultivation of Skin Epithelium of Frog.)